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Novel Strategies for the Analysis of Drugs of Abuse

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NOVEL STRATEGIES FOR THE ANALYSIS OF DRUGS OF ABUSE

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CERTIFICATE OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this thesis, that the original work is my own except as specified in acknowledgments, and that neither the thesis nor the original work contained therein has been submitted to this or any other institution for a degree.

Majed Alshamaileh

MAJED ..... (Signed)

25/05/2016 ..... (Date)

Table of Contents

List of Figures

List of Tables

List of abbreviations

Abbreviation	Term
4-FMA	4-Fluoromethamphetamine
4-MMC	Mephedrone
ACN	Acetonitrile
ADME	Absorption- distribution- metabolism- elimination:
API	Atmospheric Pressure Ionization
CI	chemical ionization
CV	Coefficient of variation
CYP450	Cytochrome P450 group of enzymes that catalyze the oxidation of organic substances
Da	Dalton
DAD	Diode array detector
E	Electrostatic field strength
EC_{50}	Effective concentration required to induce a 50% maximal effect
EI	electron ionization
EI-MS	Electron-impact Mass spectroscopy
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ER	endoplasmic reticulum
ESI	Electrospray ionization
FAB	Fast Atom Bombardment
FMOs	Flavin monoxygenase enzymatic system
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-MS	Liquid chromatography- mass spectroscopy
HPLC	High performance liquid chromatography
ICH	International conference on harmonization
IR	Infrared

IUPAC	international union of pure and applied chemistry
LC	Liquid chromatography
LC-MS	Liquid chromatography- mass spectroscopy
LC-MS/MS	Liquid chromatography-tandem mass spectroscopy
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	mass-to-charge ratio
MAOs	Monoamine Oxidase enzymatic system
MCAT	Methcathinone
MS	Mass Spectrometry
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
MXE	Methoxetamine
NADPH	Reduced Adenine Dinucleotide Phosphate
NCCD	Nomenclature Committee on Cell Death
NMR	Nuclear magnetic resonance
NPS	Novel psychoactive substances
PAR	Peak area ratio
RPC	reversed phase chromatography
RSD	Relative standard deviation
SI	Standard International
SIMS	Secondary Ion Mass Spectrometry
SPE	Solid-phase extraction
ToF	Time of Flight Mass Analyser
UHPLC	Ultra-High Performance Liquid Chromatography
UV	Ultra violet
Vis	Visible

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Abstract

The data presented in this thesis has been organized in three parts: First part included the development and validation of a quantitative HPLC-DAD analytical method of mephedrone after extraction from spiked whole blood and serum samples, alone and with methcathinone. The second part included *in vitro* metabolism of mephedrone and other NPS, which are methoxetamine and methcathinone, using an in-house prepared *in vitro* metabolic system, namely liver microsomes, followed by performing analysis for the drugs and their proposed metabolites utilizing LC-MS. Third part included *in vitro* studies of selected NPS using purchased HepaRG and hepatocytes. *In vitro* study included *in vitro* cytotoxicity studies of 4-fluoromethamphetamine, mephedrone, methoxetamine and methcathinone, and analytical studies of these drugs of abuse and their potentially produced metabolites using GC-MS.

In the first part of this thesis, a HPLC method for the analysis of mephedrone after LLE from blood matrix was developed and validated and shown to be linear with R^2> 0.995, precise with intraday and interday RSD values of 4.36 and 4.77% respectively and LOD and LOQ of 0.025 and 0.082 µg/mL respectively. Recovery percentages were low and ranged between 28-37%. Emulsion formation was the major problem effaced which negatively affected recovery and precision values. The previously developed method was optimised and fully validated for the simultaneous analysis of mephedrone and methcathinone after liquid-liquid extraction (LLE) from whole blood and serum samples. The LLE method was optimised through selection of extraction solvent and adjustment of pH values achieving the best validation parameters and minimal emulsion formation. The LLE protocol involved extraction with a mixture of dichloromethane: n-butanol (80:20 v: v) after buffering the sample with borate buffer pH=9.2 and using aniline as internal standard. The HPLC-DAD method was optimized, using reverse mode chromatography and buffered mobile phase of (acetate buffer pH 4.1: ACN – 85:15) for qualitative and quantitative analysis of these drugs in less than 10 minutes under isocratic elution and ambient temperature. The method was fully validated for both drugs and showed to be linear over the specified range of 0.1-10 µg/mL with R^2 > 0.99 for both drugs. The accuracy was assessed by calculating percentage recovery at different concentrations for

both drugs, and retained recovery percent between 84-110%. For repeatability and intermediate precision tests, RSD values were ≤ 6.73%. Specificity was assessed by good resolution between the peaks and by checking peak purities. Limit of detection and limit of quantification, calculated mathematically for both drugs either extracted from whole blood or serum samples, were 0.010-0.013 µg/ml and 0.032 - 0.043 µg/mL, respectively.

In the second part, *in vitro* studies on the metabolism of the selected NPS using pig liver microsomes and liquid chromatography-mass spectrometry (LC-MS) analysis were performed. Microsomes were prepared by a conventional ultracentrifugation method. In brief, pig liver was brought freshly from local abattoir, sliced into small pieces, homogenised and ultra-centrifuged to produce microsomes and S9 fractions. Produced microsomes were incubated with the drugs of interest under optimised conditions and followed by analysis utilizing LC-MS for the detection of the drugs and the potentially produced metabolites. It was possible to detect two metabolites of the drug mephedrone, hydroxytolyl-mephedrone and nor-dihydro mephedrone. For MXE, one metabolite produced by the O-demethylation was detected and it identity confirmed by MS/MS study to be o-desmethyl-MXE. Another metabolite was detected is suggestively produced by the reduction of the ketone moiety to produce dihydro-MXE or by two steps of O-demethylation and hydroxylation to produce O-desmethyl –hydroxy-MXE. However, due to low intensity signal recorded, MS/MS study was not conclusive for the identity of the molecule

In the third part, two types of hepatocytes were used for the study of the metabolism and cytotoxicity of the selected NPS - Mephedrone, Methoxetamine, Methcathinone and 4-Fluoromethamphetamine. Studying the metabolism of selected NPS followed utilizing HepaRG™ followed by GC-MS analysis, it was possible to detect new peaks in the chromatograms of mephedrone and methcathinone which is suggestively the product of N-demethylation. However, it was not possible to detect any new peaks in the chromatograms of methoxetamine nor 4-flouromethamphetamine. The cytotoxicity study utilizing HepaRG cell line showed that these drugs have cytotoxic effects causing *in vitro* cell death, within the specified range of $4.0x10^{-2}$-$1.6x10^{1}$ mM. These drugs were able to cause 43-83%

cell death, and EC_{50} values were 0.2323-0.6297 mM. The most potent drug was 4-fluoromethamphetamine, while mephedrone showed the least biological effect to produce.

Chapter 1 Introduction

1.1. Aims and objectives

A huge number of novel psychoactive substances (NPS) have appeared recently on the market. Those NPS are being easily obtained via Internet websites or through so-called 'headshops' or 'smart shops'. These substances are sold labelled 'not for human consumption'. However, they are purposefully marketed as replacements for illegal drugs. In fact, in most cases these NPS are very closely structurally related to controlled psychoactive molecules in order to create alternative psychoactive compounds. Due to this structural relationship, these NPS may show similar clinical and toxic effects similar to controlled ones. The misleading idea of being 'safe' drugs because of their legality in many countries, strongly increased the use of these substances, especially among young age groups of 15-64 years old. Cathinone derivatives, along with synthetic cannabinoids, represent currently about two-thirds of all substances notified since 2005 (Kelly, 2011; Araújo et al., 2014). The abuse of novel psychoactive substances (NPS) have been a matter of concern for public health, many cases of death because of intoxication with these chemicals have been recently reported. However, this phenomenon is undervalued in the EU because the outdated toxicological screening tests do not cover the wide range of these novel molecules. The rapidly increasing number of constantly varying NPS makes their identification and the study of their analytical and toxicological profile an extremely difficult task, especially when standards are not available (Rosner et al., 2005; Kelly, 2011; Favretto et al., 2013; Araújo et al., 2014).

Due to these restrictions, the aims of the current work were developing novel approaches for the analysis of drugs of abuse that keep in pace with rapidly appearing 'NPS' and able to provide real time information about the toxicity and risks associated with these drugs.

The availability of HPLC instruments and DAD type of detectors is advantageous as a preliminary quantification tool for NPS. HPLC-DAD methods are quiet easy to develop, specific and robust for identification and quantification NPS. The aim of the first part of this thesis research was to develop validated methods for the analysis of only mephedrone, with other drugs, in presence of adulterants

and related cathinones after extraction from biological samples. This method can be helpful for future analysis of other appearing NPS both qualitatively and quantitatively.

The limited data about metabolic studies of NPS is another concern while researching NPS. The second part of this thesis research started with limited data available about the previously studied mephedrone and another emerging NPS, MXE. Due to ethical limitation of researching these NPS *in vivo*, either in clinical setup or using animal models, *in vitro* approaches were selected for metabolic studies of NPS. Though *in vitro* studies are well established in medicinal drugs industry, the use of *in vitro* models is limited in the field of forensic study of drugs of abuse in general. *In vitro* biological studies have many advantages over *in vivo* studies, such as relatively lower cost, effort and time. Additionally, it fulfils the ethical requirements with minimal harm to animals. Consequently, the aim at this stage of study was to develop a satisfactory *in vitro* metabolizing model, by preparing microsomes from slaughtered pig liver cells and using these microsomes for metabolizing the selected NPS. This *in vitro* studies are followed by developing an analytical method applying the power of the modern instruments of chromatography coupled to mass spectrometry in order to detect these drugs and their potential metabolites.

In the third part of the work, the aim was to develop another *in vitro* model using human cells for a metabolizing system, hepatocytes. The aim was to research any variation between metabolising capacity of different *in vitro* system of different sources. *In vitro* studies using hepatocytes was followed by analysis of the selected NPS using GC-MS together with cytotoxicity studies about the impact of this selected NPS on the cell viability and if any relation existed between the type and amount of produced metabolites and any toxic effects observed.

To the best of knowledge, limited data are still available about metabolism of the selected NPS, namely Mephedrone, Methoxetamine, Methcathinone and 4-Fluoromethamphetamine, and other traded NPS. The currently available data do not fulfil the critical need for analysis of these significant drugs of abuse.

1.2. Epidemiology of drug abuse

Drug abuse can be defined as the use of a pharmacologically active substances for non-medical purposes. The cultural problem of drugs abuse is not a modern dilemma; as the history of drug intake can be traced from the days of ancient Greece, where from that time to the present, religious and legal attempts have been performed in order to control drug abuse and trade (Lambert, 1998; Compton et al., 2005; Sloboda, 2005).

Drug abuse is worldwide problem, though there are variable trends between regions, age groups and genders. In general, men are at least two times more likely than women to have drugs of abuse. Worldwide, about 5% (243 million) of the population of the age group 15-64 years had used, at least once, one or more of drugs of abuse, as being estimated in 2012. However, about 27 million of the world population regularly use drugs of abuse (UNODC, 2014).

Drugs of abuse are of critical concern for the public and the authorities because of the devastating effect they have, where drug related death is the most dangerous effect. Worldwide, it is estimated that in 2012 drugs were the cause of death of about 183,000 people in the 15-64 years' age group (UNODC, 2014).

The National Program on Substance Abuse Deaths (NPSAD) publish an annual report about drug related deaths all over UK and islands. The data collected are reported by coroners, police forces, statistic and research agencies. There are minor differences in the inclusion criteria for the drug related deaths between geographical areas. In general , for the 2013 annual report, a death fell within the NPSAD criteria for drug related deaths when: one or more psychoactive substance directly associated with death: history of abuse or dependence : presence of controlled substances in post-mortem samples or when the death is directly caused by drugs but with no investigation According to the NPSAD drugs were the cause of death in at least 2478 cases in 2012 compared to at least 2952 cases in 2011 all over the UK and islands (Ghodse et al., 2013). According to study conducted by Bargagli et al. during 1990-1998 about the estimated mortality rates of opiate users in Amsterdam, Dublin, Barcelona, Lisbon, Rome, Vienna, Denmark and London, opiates were the cause of death in

10-20% of mortalities in the 15-49 years old age group in these eight European countries. The study showed that mortality rate within opiate users is 5-54 times higher than general population (Bargagli et al., 2006).

Non-fatal cases of drug abuse are also important. It is estimated that in Europe, for each one fatal drugs related death there are another 20-25 non-fatal cases of overdose. The importance of these non-fatal cases is that they contribute to morbidity that may need hospitalization due to different side effects of drugs of abuse. In more serious cases they may end with some sort of disability, mainly due to brain damage. In addition, drug abuse is negatively affecting the quality of life and it is a major cause of either long or short term health impairment. For example, the prevalence of transmitted infection, mainly HIV, is estimated to be 22-50 higher between drug abusers compared to the general population (UNODC, 2014).

The adverse effects of drug abuse involve not only the individuals who abuse drugs, but also their families and friends, government resources and the whole society. Families will be affected by the feeling of guilt and stigmatization by the community as well as financial impacts in terms of cost. The attempt by drug abusers to manage their drugs costs by stealing from families is an example of financial cost. The social impact relies on the cost to cover the care and rehabilitation costs. Drug users need extensive medical treatment; some may abuse or neglect their families; some may commit crimes including domestic violence, assault, and theft. As an example, the economic cost of illicit drug use on American Society in the year 2007 was about $193.1bilion. In comparison to other societal costs, it is more than the estimated costs of diabetes in the year 2008 which were estimated more than $174 billion, and more than the medical costs associated with obesity in the year 2008 which were estimated more than $147 billion (National Drug Intelligence Centre, 2011).

In UK, the ‘Misuse of Drugs Act 1971’ governs the legal status of drugs. The ‘Misuse of Drugs Act 1971’ categorizes controlled drugs into three classes, A, B and C. Under the force of this act the importation, exportation, production, possession and supply of controlled drugs is completely restricted (Parliament of the United Kingdom, 1971). Although the primary act was implemented in

1971, the Advisory Council on the Misuse of Drugs regularly updates the legal framework in response to national and worldwide drug threats.

1.3. Novel Psychoactive substances

Novel Psychoactive Substances (NPS) are a variable class of compounds, rapidly appearing in the illicit drug market that show significant psychoactive effects. These substances often have no limitations on commercial delivery as they claim to contain 'non-illegal' compounds. It is estimated that 20 or more NPS appear annually, either through the internet or through what is known as 'smart shops.'

In general, NPS can be considered into four categories: substances available under trade names without indication of their contents, related medicinal chemicals, natural herbal extracts or fungal products and substances specifically designed with minor alterations to simulate the effect and/or the chemical structure of known controlled drugs (ACMD, 2011).

The term 'Designer Drugs' is mainly used to describe one category of NPS, which is produced by performing minor alterations to one or more functional group of a chemical with known pharmacological activity, to avoid the legal regulations and to produce more effective substances (Henderson, 1988; Christophersen, 2000; Camilleri et al., 2010). 'Designer drugs' are usually synthesized, distributed and used in low levels and within small sub-populations, which makes their detection and control challenging for authorities and scientists (Wills, 2005; EMCDDA, 2010).

Characteristic examples of NPS are synthetic derivatives of cathinones and synthetic cannabinoids. The expressions 'Designer Drugs' or 'legal Highs' were used in literature interchangeably to refer to NPS, while the term NPS is an expansive descriptive term for these versatile groups of drugs. In fact, it is advised not to use the term 'Legal Highs' in order to avoid confusing the public as this term may reflect positive euphoric effects in addition to the legality of these substances. Also, the use of the term 'legal highs' to describe these groups of drugs, can give a false impression about the safety of these drugs. Legal Highs are usually traded as research chemicals, bath salts or plant food, with some mentioning clearly the contents. However, it has been reported that legal highs available on the

internet do not always contain the labelled drug, but caffeine or any other controlled drug (EMCDDA, 2011; Baron et al., 2011; Prosser and Nelson, 2012; Favretto et al., 2013).

During 2009, 24 new synthetic substances were reported for the first time in European Union (EU), and by mid of July 2010; another 15 new synthetic substances were added to the list. In 2014, additional 101 novel designer drugs were reported, of which 31 drugs were cathinones. Therefore, the entire number of designer drugs examined by the European Monitoring Centre for Drugs and Drug Addiction has exceeded 450 (EMCDDA, 2015). Due to this rapidly appearance of NPS, and mainly the lack of standard references, the development of new analytical methods to detect 'Designer Drugs' is a significant challenge for the forensic scientists.

The synthetic cathinones are one of the major groups of designer drugs. Synthetic cathinones are central nervous system stimulants, and are abused for their stimulant Amphetamine-like effects including enhanced energy and increased libido. They are chemically similar to Cathinone ((S)-2-amino-1-phenyl-1-propanone), a naturally occurring stimulant alkaloid with beta-ketone skeleton found in the fresh leaves of the Catha edulis (Khat) plant. Leaves of Khat have been chewed since the tenth century in some African countries and very widely in Yemen. In UK, cathinone is classified C drug in Schedule 1 of the UK 1971 Misuse of Drugs Act. Bupropion is the only synthesized cathinone-derivative that was approved for therapeutic purposes in Europe; and it is medically indicated now as an antidepressant and as an aid in smoking-cessation (Gibbons and Zloh, 2010; Winstock and Wilkins, 2011; Kavanagh et al., 2013).

Synthetic cathinones are sold mainly as blends of white powder, crystalline salts and liquid forms, though oral tablet and capsules preparation are also available. Current studies show that the main mechanism of action of synthetic cathinones is the dysfunction of central monoamine systems that apparently cause their interactive properties (Prosser and Nelson, 2012).

The beta-ketoamphetamines, with methcathinone and mephedrone as better-known members of this family, as examples of synthetic cathinones, have rapidly entered the drug market in the last few decades (EMCDDA, 2010; Grueninger and Englert, 2011). Methcathinone and mephedrone were

reported to be synthesized in 1929. Their abuse started in the early 1990s for Methcathinone and the early 2000s in the case of mephedrone. Though little data are available on the clinical and toxicological profiles of the synthetic cathinone derivatives, the reported side effects of these derivatives are similar to those related to cathinone intake: elevated blood pressure, increased heart rate, probable risk of myocardial infarction and duodenal ulcers (Gebissa, 2010; Al-Motarreb et al., 2010). In general, synthetic cathinones have nearly similar clinical and toxicological effects to each other and naturally occurring cathinone and amphetamines, which can be explained by the structural similarity of these drugs (Figure 1)

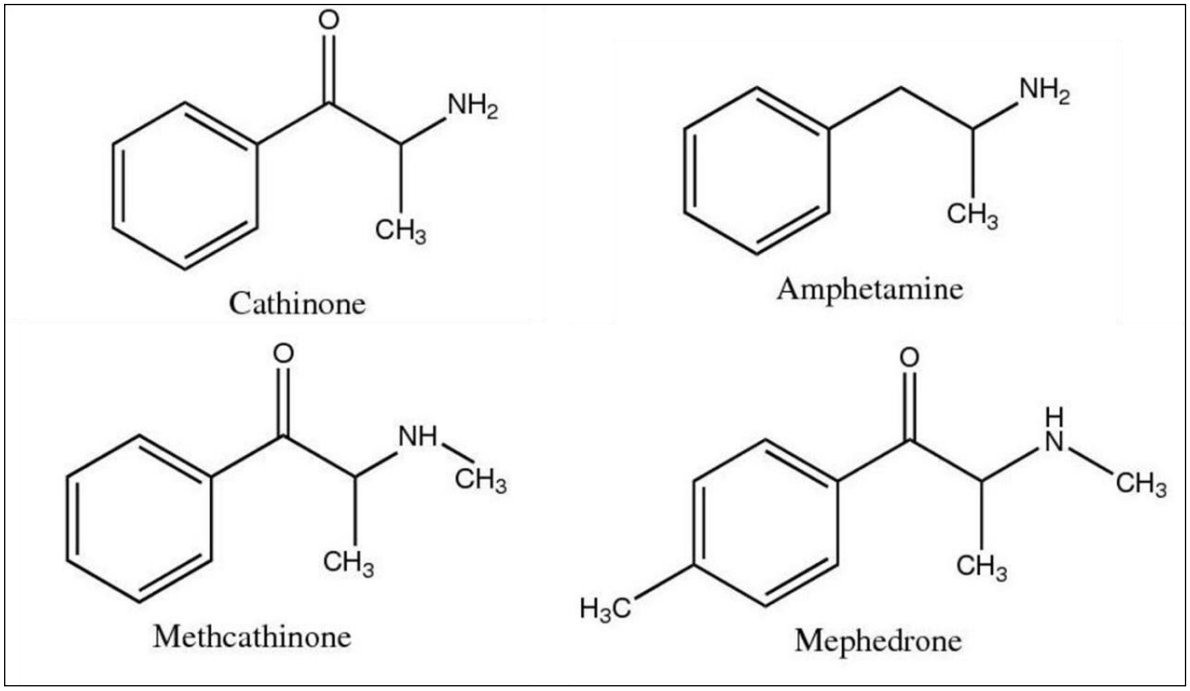


Figure 1: Structural similarity of amphetamine, cathinone, methcathinone and mephedrone

1.3.1. Mephedrone

Mephedrone, (4-methylmethcathinone, IUPAC: 2-Methylamino-1-p-tolyl-propan-1-one) is a synthetic cathinone derivative (see Figure 1 above), where the history of mephedrone synthesis dates back to 1929, when it was reported by Saem de Burnaga Sanchez (Europol-EMCDDA, 2010). No much data is available about the clinical or toxic effects of mephedrone, and it has never been licensed as a medicine anytime since its first appearance in the illicit drug market in 2007 (WHO, 2014).

Mephedrone started to appear online and become available in 2007 (Schifano et al., 2010), and it was traded and consumed legally in many countries. It was late April 2010 when mephedrone was added to the list of the illegal drugs of abuse in UK, after some fatalities were related to the drug. By the end of July 2010, mephedrone was also identified in at least 38 drug-related fatalities, whereby in two cases mephedrone was the only detected drug (Morris, 2010)

Mephedrone comes in forms of powder, tablets or capsules. It may be swallowed, injected, snorted, smoked or even taken rectally. The most common effects reported with mephedrone intake include euphoria, tachycardia, agitation, anxiety and tremor. These effects are similar to the typical effects of cathinone, which have similar effects to the amphetamines group which include also elevated blood pressure, increased heart rate, probable risk of myocardial infarction and duodenal ulcers (Bentur et al., 2008; James et al., 2010; Gebissa, 2010; Al-Motarreb et al., 2010; Grueninger and Englert, 2011).

Since its appearance in 2007, work concerning mephedrone has been related to developing analytical methods for the identification of the drug and its metabolites in biological samples utilizing different analytical instrumentation. For example, these methods mainly utilized LC-MS/MS, GC-MS (Baron et al., 2011; Jankovics et al., 2011; Sorensen, 2011) while some utilized HPLC-DAD for qualitative and quantitative studies (Singh et al., 2010; Santali et al., 2011).

1.3.2. Methcathinone

Methcathinone (IUPAC: 2-Methylamino-1-phenyl-propan-1-one) is a methyl derivative of cathinone which has related chemical structure, and hence similar clinical effects, to amphetamine, to mephedrone and to cathinone, with comparable sympathomimetic effects (see Figure 1 above). History of synthesis of methcathinone dates back, similar to mephedrone, to the 1920s. The first case of methcathinone intoxication was described more than 20 years ago, but nowadays, there have been significant increases in both the number of people who use this drug and the total number of cases of intoxication. As in the administration of other synthetic cathinones, the most reported route of methcathinone intake is through snorting, though oral and intravenous injection are also used.

Clinical effects are also similar to cathinones, amphetamine and related drug and include euphoria, increased alertness, increased heart rate and blood pressure (Baumann et al., 2013). There are publications dealing with analysis of methcathinone utilizing, for example, HPLC-DAD (Golasik et al., 2014) or GC-MS (Beyer et al., 2007; Sorensen, 2011).

1.3.3. 4-Fluoromethamphetamine

4-Fluoromethamphetamine (4-FMA, IUPAC: [2-(4-Fluoro-phenyl)-1-methyl-ethyl]-methyl-amine), is a central nervous system stimulant related to methamphetamine (Figure 2). It is found to increase the effectiveness and duration of action of methamphetamine by being an inhibitor of the enzyme CYP450, so it reduces its metabolism. Generally, the replacement of a hydrogen atom by fluorine increases the lipophilicity, and so increases the drug absorption into biological membranes and its passing through the blood brain barrier of central nervous system. Additionally, small covalent radius of fluorine assists binding with biological receptors. Rosner et al., 2005; Taniguchi et al., 2010; Taniguchi et al., 2013). There is no sufficient data yet known about the metabolism, toxicity and clinical effects of 4-FMA**.**

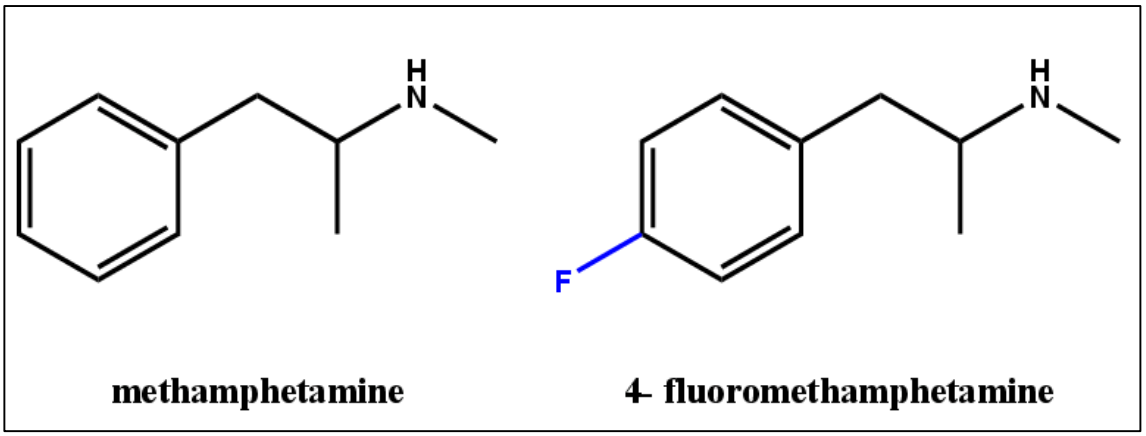


Figure 2: Chemical structure of methamphetamine and 4-fluoromethamphetamine

1.3.4. Methoxetamine

Methoxetamine (MXE, IUPAC: 2-Ethylamino-2-(3-methoxy-phenyl)-cyclohexanone) is a dissociative anaesthetic that has been traded as a designer drug. It is distributed as a white powder under different names, such as Kmax, M-ket and Mexxy. MXE is synthetically derived from

ketamine, and shares the same mechanisms of action and clinical effects, with even stronger and longer psychoactive effects than ketamine. The first case of methoxetamine abuse that is analytically confirmed was reported in 2012. MXE can be administrated sublingually, nasally or parentally, and the reported symptoms of MXE include tachycardia, hypertension, confusion, agitation, hallucinations, mydriasis, and nystagmus. (Hofer et al., 2012; Coppola and Mondola, 2012).

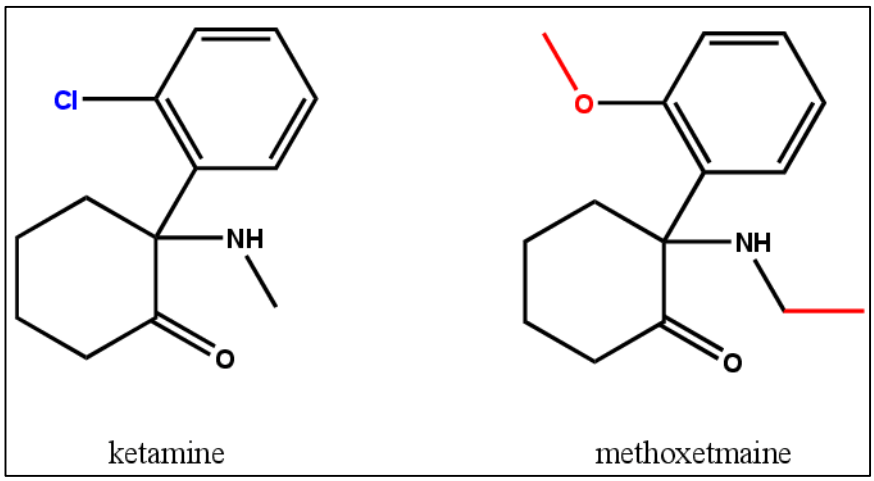


Figure 3: Chemical structure of Ketamine and methoxetamine

As with all other NPS, limited data has been found on the metabolic profile of MXE, though some clinical data has been reported in the literature in the last couple of years (Shields, Dargan et al. 2012, Hofer, Grager et al. 2012). Little information is available about the study of the metabolism of MXE from an analytical point of view. To the best of knowledge, only two recent papers on *in vitro* and *in vivo* studies (Meyer et al., 2013; Menzies et al., 2013). The *in vivo* study by Meyer et al. investigated the metabolism of MXE using male rats by administration of single dose MXE through gastric route and then collecting urine and faeces separately over 24 hours, in addition to urine samples received by the authors' laboratory for toxicological analysis, and the prepared samples were then analysed utilizing GC-MS and LC-MS (Meyer et al., 2013). A recent literature review also yielded another published work about analytical findings after acute MXE intoxication (Imbert et al., 2014). There are no other available data about the acute effects of MXE exposure, and only two more available studies about the chronic effects of MXE exposure using animal models (Wood et al., 2012; Yew et

al., 2013). The restricted information about the toxicological profile of MXE acted as an incentive to developing approaches for the production and identification of its metabolites.

1.4. Biotransformation of drugs

The biotransformation of exogenous chemicals is a main mechanism for drug elimination, and the products of the biotransformation mechanism - the metabolites - are generally more polar and water soluble than the parent chemical which makes their excretion easier, and so prevents the build-up of these exogenous, usually toxic, chemicals in the body. However, sometimes biotransformation does not detoxify these exogenous chemicals but produce metabolites that are more toxic than the parent molecule. Almost every chemical entering the body undergoes biotransformation reactions catalysed by one or, most of the time, more than one enzymatic system. The type of the catalysing enzymatic system and the metabolic pathway the chemical will go through are highly dependent on its chemical structure. Chemicals that are structurally similar tend to share the general metabolic pathway and the enzymatic group that catalyses their biotransformation.

Typically, after absorption (A) of any chemical through one or more of the body systems, it will go into a detoxification process. The detoxification process starts by distribution (D) of the chemical in blood, body fluids and target organs followed by the metabolism (M) of the chemical in different body organs. The last stage is the elimination (E) of the chemical and/or its metabolites as body waste products. This is known as ADME model, a very important concept in the drug industry, pharmacokinetic and toxicological studies. Metabolism, the (M) part of ADME model, is a major pathway for detoxification of the chemicals in the body, where it is responsible for about 75% of the whole process, either partially or completely. In general, the aim of toxicokinetics and pharmacokinetics studies of a chemical is the identification of its ADME parameters (Figure 4).

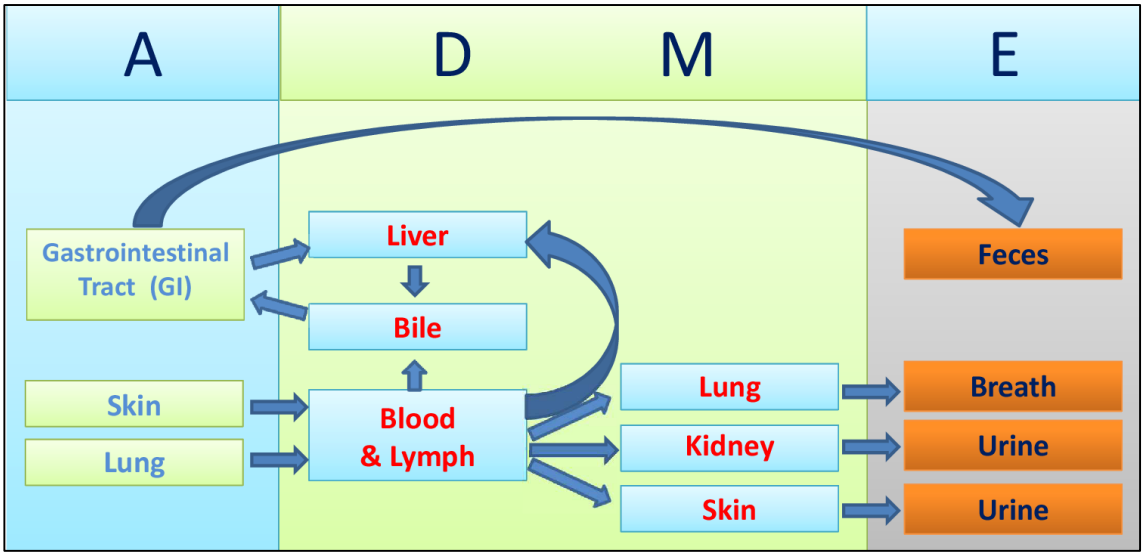


Figure 4:ADME model of the biotransformation of drugs

1.4.1. Metabolism

Chemicals entering the body, mainly through gastrointestinal system are metabolized by enzymes, which may decrease, increase or totally change their action. Toxic materials can be detoxified while nontoxic drugs can be converted into toxic metabolites.

Classically, drug metabolism includes two phases; phase I in which nonpolar molecules are changed to polar ones, and phase II (conjugation) where glucuronic acid, sulfate, methyl or acetyl moieties are added to drugs molecules (Asha and Vidyavathi, 2010). However, Phase III is now added to the classical description of chemicals metabolism which is responsible for the transportation of the molecules produced from metabolic process from the extracellular into the extracellular space utilizing energy and specific transport system (Coleman, 2010).

Metabolism takes place anywhere in the body, e.g. in the intestines, in the liver and in the kidney. However, liver is the major organ responsible for the metabolism of exogenous compounds, greatly reinforced by its anatomical position between systemic circulation and the gastrointestinal tract and its rich content of metabolizing enzymes, mainly the cytochrome P450 enzymatic system (CYP450). Liver is the second largest organ in the body, weighing in an adult human in average 1500 gm, which is about 2% of the total body weight. In addition to its major function as a detoxifying organ, liver has other important functions, which include: protein synthesis, bile production and hormone

production. Chemicals entering the body through the gastro intestinal tract are transported directly into the liver via the hepatic portal vein, a major vein in the body, which drains venous blood from the gastro intestinal organs. The main function of metabolism is to change the physicochemical properties of the exogenous compounds from less water-soluble to more water-soluble to be easily excreted in urine.

Usually metabolism goes in order from Phase I-III, but some chemicals may go through one phase without being preceded or followed by the other. Phase I is mainly an oxidative reaction catalysed by the CYP450 enzyme family group, though other reduction and hydrolysis reactions are catalysed by other enzyme families, and are included in 'Phase I' reactions.

Phase II reactions are mainly conjugative processes, where sugars, amino acids or salts are conjugated to the chemical, again with the aim to make the chemical more water soluble and easy to eliminate. Phase III is about the removal of the hydrophilic conjugates produced from phase II metabolism from inside the metabolizing cells through the lipophilic cell membrane to the extracellular fluid and then back to the bloodstream until they reach kidneys to be excreted (Coleman, 2010).

1.4.1.1. Phase I Metabolism

The aim of phase I metabolism is to add polar reactive groups into the structure of the xenobiotic, that may be performed by oxidation, hydrolysis, reduction, cyclization or decyclization. After phase I, if the generated metabolites are adequately polar, they can be excreted without undergoing phase II metabolism. Oxidation reactions are mainly catalysed by Cytochrome P450 monooxygenases. However, Flavin monoxygenase enzymatic system (FMOs), alcohol and aldehyde dehydrogenases, Monoamine oxidases and peroxidases can catalyse these oxidation reactions. The NADPH-cytochrome P450 reductase is the enzyme mainly responsible for reduction reactions. Hydrolysis reactions are catalysed by amidase, epoxide hydrolases or esterase enzymes. The physicochemical properties of xenobiotic compounds are changed after metabolism, and the new chemicals – metabolites – will have different structure after undergoing metabolic reactions. This is a very

important process as it is producing metabolites with target effects (i.e.: activation) or producing metabolites with no, less or benign effects; i.e.: detoxification (Woolf, 1999; Guengerich, 2001; Foye et al., 2012).

The start of the research with CYP450 dates back to the 1940s, through *in vitro* studies on the metabolism of drugs. Spectral observation, the role as oxidase in the electron transport system, separation and purification of the P450 enzymes were the major Followed by several studies including biochemical and biophysical work with different species of the P450 enzymatic systems (Guengerich, 2001; Guengerich, 2007; Coleman, 2010).

CYP450 are a group of enzymes with approximate molecular Weight of 50000 Da that share the core structure and mode of action, and they are responsible for more than 75% of chemicals metabolism, making them a principal player in the metabolism in living bodies (Woolf, 1999; Foye et al., 2012). So far, more than 7700 CYP450 enzymes have been identified, out of which about 57 are human and only 15 out of these human CYP450 are known to be responsible for metabolism of chemicals (Coleman, 2010).

The general structure of the CYP450 is a central haem iron group supported by a protein frame known as ferriprotoporphyrin-9 (Figure 3). This is a common core structure of CYP450 and the active site of its enzymatic oxidative function, and it is a common supportive structure in other enzymes containing the haem core (e.g.: haemoglobin, myoglobin). Through and over the core structure of the haem and F9 support, helices run as covering structures and help to add flexibility to the core active site. The active site of the CYP450 enzyme is the area where the chemicals are catalysed. However, though CYP450 enzymes share a common structure and general function, there is an extreme difference in the specificity for the chemicals between them, even within the same family member (Coleman, 2010).

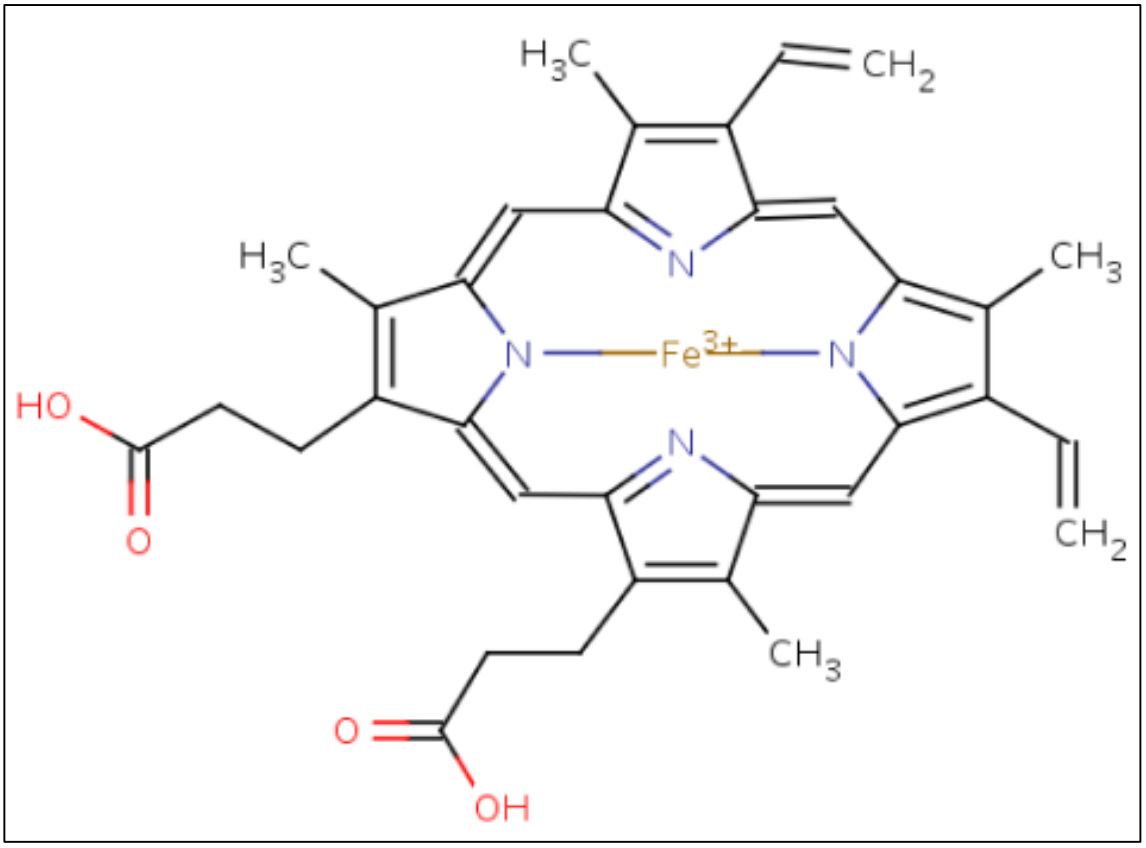


Figure 5: General structure of ferriprotoporphyrin-9 support

Cytochromes P450 are systematically classified into families, where CYP450 of the same family share 40% of the amino acid structure and each family is sub classified into sub families where the CYP450 of the same subfamily share 55% of the amino acid structure. The CYP450 nomenclature is of four parts: the prefix (CYP) followed by number in reference to the family, letter in reference to the subfamily and ends by a number in reference to the isoform of the same enzyme. Despite the various numbers of CYP450 and their isoforms, mainly CYP1, CYP2 and CYP3 families are involved in chemicals metabolism in humans, and it is alleged that 90% of the known chemicals metabolism take place by one of these five isoforms: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Woolf, 1999; Guengerich, 2001; Coleman, 2010; Foye et al., 2012).

The CYP3A subfamily enzymes are of significant importance as they are one of the most abundant CYP450 enzymes in the human body and being responsible for the metabolism of wide range of chemicals in terms of types and molecular weight (i.e. they can metabolize large number of different types of molecules and chemicals either with high or low molecular weight). More than 50% of the

hepatic content of the CYP450 enzymes alone belongs to the CYP3A subfamily, mostly one of the two isoforms: CYP3A4 and CYP3A5, where they represent around 30% of the total CYP450 contents of the liver. In addition, those two specific isoforms, CYP3A4 and CYP3A5, represent a significant amount of the total content of CYP450 in the lung (Woolf, 1999; Coleman, 2010; Foye et al., 2012).

Flavin Monoxygenases (FMOs) are another important oxidation system, mainly responsible for the metabolism of endogenous chemicals that are widely available in the human body. They have comparatively similar nomenclature system like CYP450, relying on the similarity of the amino acids sequence within one family or subfamily. FMO-3 is the most abundant enzyme of the FMOs in human liver, while FMO-1 is the most abundant in the liver of experimental animals. This significant difference in type and abundance is very important when transferring research results regarding FMOs from animals to humans. It is believed that FMO-3 is expressed a little less than two-thirds the rate of expression of CYP3A4; the most abundant CYP450 enzyme in human liver (Woolf, 1999; Coleman, 2010; Foye et al., 2012).

Monoamine Oxidase (MAOs) is another significant enzymatic system, which is not only responsible for the metabolism of exogenous chemical, but also for the metabolism of endogenous chemicals. There are only two forms of the MAOs, A and B, both having similar functions and present almost in every part of the human body (Woolf, 1999; Coleman, 2010; Foye et al., 2012).

1.4.1.2. Phase II metabolism

Phase II reactions are mainly conjugation reactions that add an endogenous substance to the parent or processed drug. This conjugation reaction needs the compound to have a functional group (i.e. hydroxyl group) to act as the conjugation site. This functional group maybe already present, which make the conjugation process occurs directly or may be produced by one or more of the metabolic process (i.e. phase I reactions). Phase II reactions include sulphation, glucuronidation and methylation.

As it was discussed earlier, the main aims of metabolism are to alter the effect of chemicals by either activation, deactivation or modification. Make the chemical itself, or produce metabolites that will be more water-soluble to ease its excretion in body wastes.

The first aim is usually achieved by phase I enzymatic reactions while phase II is responsible mainly for achieving the second aim. The CYP450 and other enzymatic systems typically change the polarity of every chemical more toward hydrophilicity, but not enough for the processed chemical to be excreted in polar body wastes, e.g.: bile, urine and sweat. This problem, most of the time, is solved by phase II reaction (Coleman, 2010; Foye et al., 2012).

Glucuronidation is the major Phase II reactions, which is catalysed by UDP-glucuronosyltransferases (UGTs), an enzyme with more than one known isoforms in human. The glucuronidation reaction involves the relocation of glucuronic acid to a chemical functional group leading to increasing in the polarity of the chemical, which in result increases the chemical solubility in water facilitating its excretion. The liver, as it is in Phase I reactions, is a major site in humans for glucuronidation. Glucuronidation takes place in other sites of the body, and for some drugs, some sites are preferred for glucuronidation. Sulphonation is another important Phase II reaction catalysed by sulphotransferases (Woolf, 1999; Coleman, 2010; Foye et al., 2012).

1.4.1.3. Phase III metabolism

In general, the aim of biotransformation is to detoxify the effects of the exogenous chemical, and to change its chemical structure into a more water soluble one to facilitate its excretion, mainly through urine. This is achieved by Phase I and Phase II reactions. However, those reactions take place in the intracellular space, i.e. inside the cell, and the produced chemicals, i.e. the metabolites and their conjugates, needs to be transferred into the extracellular space, i.e. outside the cell, against a concentration gradient to enter the blood stream and ultimately be filtered by the kidneys.

The structure of the cell membrane, which is composed mainly of phospholipids, is relatively impermeable for large and water-soluble molecules. The products of Phase I and Phase II metabolism are relatively water soluble and large molecules that will then be entrapped inside the cell unless they

are actively transferred from the intracellular space into the extracellular space. Phase III metabolism refers mainly to the transport process of the metabolic products from the intracellular space into the extracellular space. This process needs a specific transport system in addition to energy. Phase III is important because the hydrophilic products of phase II metabolism inside the cells, may either undergo hydrolysis to give the original xenobiotic, inhibit the conjugating enzymes or even they can be themselves cell-toxic (Coleman, 2010).

1.5. *in vitro* models for the study of drugs of abuse

Historically, animal models have been used for decades aimed at studying the effect of chemicals as a predictive *in vivo* system, but sometimes they produce unreliable prediction of human metabolism and toxicity (Olson et al., 2000; Turpeinen et al., 2009). Russel and Burch suggested- for the first time the concept of the 3R's, standing for Replacement, Reduction and Refinement, suggesting replacing the living animals by non-conscious alternatives, reducing the number of used animals to the minimum to obtaining same or comparable results and refining methods that would causes minimal pain and distress for the used animal models (Flecknell, 2002).

In order to bring the 3R's concept into application, the development of *in vitro* systems to study the effect of drugs has been considered as an alternative for the animal models, drawing the attention of researchers and scientists. However, it is thought that full replacement of the animal models is not yet possible. The search for alternatives is very important because, most of the time, local regulations have restrictions for using animals, in addition to the expense of using animal models. Furthermore, there is always a query about the assumption that the chosen animal model will adequately predict those effects in humans. Frequent interspecies difference in pharmacokinetic studies may explain low prediction rates of drug-induced toxicity in humans when data are collected from animal studies (Olson et al., 2000; Flecknell, 2002; Guguen-Guillouzo and Guillouzo, 2010; Adler et al., 2011; Anthérieu et al., 2012).

There are diverse available *in vitro* models, such as whole organs, tissue slice, cells and subcellular fractions. Utilizing cells or subcellular fractions makes the study system easier, also permitting the assessment of a specific reaction-related mechanism (Chiu, 1993; Olson et al., 2000).

There is little information on methods mainly using computer-based approaches, where the basic theory behind such methods is to link the toxic and/or clinical effect to molecular parameters associated with the chemical structure. This is a predictive tool that may be transferred later into testing method, depending on the type of the information to be obtained (Davila et al., 1998; Flecknell, 2002; Turpeinen et al., 2005; Adler et al., 2011).

The clinical effects of drugs are mainly dependent on its absorption/ distribution/ metabolism/ excretion parameters, and so the metabolic steps that it goes through would affect its behaviour (Turpeinen et al., 2005). Liver preparations are the most commonly used metabolic and toxicological *in vitro* models, e.g. Hepatocytes, cytosol, S9 fractions, HepaRG cell lines and microsomes (Asha and Vidyavathi, 2010).

In vitro approaches have been considered as one of the preferred methods for studying the metabolism of NPS as clinical studies using humans or animals, to a lesser extent, have ethical implications. Additionally, *in vitro* drug metabolism methods are generally easier and more rapid to apply than *in vivo* approaches. *In vitro* drug metabolism studies using microsomes are more widely used in the pharmaceutical industry than to study the metabolic profile of new emerging drugs of abuse.

The reason behind choosing freshly prepared pig liver for the preparation of microsomes and S9 fraction for *in vitro* metabolic models of NPSs of interest was its similarity in size and physiology with that of humans. In addition, a similar distribution of different families of CYP450 enzymes between pigs and humans can be found. Collectively, these factors can be helpful in increasing the degree of reliability of the proposed metabolic procedure (Puccinelli et al., 2011; Achour et al., 2011).

The liver S9 fraction covers both cytosolic and microsomal fractions. Exogenous cofactors such as a NADPH-regenerating system is also needed to supply the energy for the CYP enzymes. S9 fractions comprise both phase I and phase II activities, however, they are poor in enzymes if compared to microsomes (Sepuri Asha, 2010).

1.5.1. Microsomes

In 1936, Potter & Elvehjem improved procedures of preparing tissue homogenates. Liver homogenates were then prepared and were considered as successful *in vitro* metabolic systems as they contain both phase I and phase II enzymes. Consequently, differential centrifugation methods were developed enabling the preparation of subcellular fractions such as microsomes (Potter and Elvehjem, 1936; Ekins et al., 2000). Microsomes are vesicle-like artefacts that are made from portions of endoplasmic reticulum (ER) after disintegrating eukaryotic cells; they can be concentrated and separated from other cellular debris by differential centrifugation (Houston, 1994; Nakamura et al., 2007).

Liver microsomes are clinically successful *in vitro* models for the assessment of drug metabolism and one of the most commonly used approaches. In addition, using microsomes is a relatively cheap, simple and easy method and the prepared microsomes can be stored in frozen form for years. On the other hand, they have many drawbacks, being unsuitable for quantitative *in vivo* human assessments of metabolism and not containing all of the enzymes and cytosolic cofactors. Thus, exogenous cofactors consisting of a NADPH-regenerating system (phase I oxidation) or uridine-5′-diphospho-α-D-glucuronic acid (UDPGA; phase II glucuronidation) are needed to activate microsomes. Microsomal *in vitro* models have significant variations depending on the liver donor and the incubation settings such as pH, ionic strength, organic solvents (Brandon et al., 2003; Asha and Vidyavathi, 2010).

1.5.2. Primary Human Hepatocytes

Primary human hepatocytes are one of the recently used *in vitro* metabolizing systems, and still the standard model to mimic *in vivo* pharmacokinetics of drugs and their toxicity in human liver. In fact,

it is well established in the scientific community that primary human hepatocytes are the best source for primary studies of drugs metabolism which make them a very important *in vitro* model for drug pharmacokinetics study, mainly for metabolic and toxicity studies (Guillouzo et al., 2007).

Primary human hepatocytes, in current times, are isolated from surgically dissected liver fragments because of tumour or other liver diseases which results in deficiency of some liver specific functions, especially P450 enzymatic activity. In general, primary hepatocytes have limited activity and wide variation in P450 levels. Additionally, primary human hepatocytes usually lose their metabolizing function quickly, especially when they are in the suspension form as they lose their naturally occurring polarity.

Use of primary human hepatocytes is affected by many limitations, such as the insufficiency of appropriate human liver samples and the variability observed when the cells are prepared from different human donors. *In vivo* hepatocytes are polarized two-sided cells, they have a sinusoidal side (blood) and canalicular side (bile) and they become nonpolar when are isolated from liver and re-suspended. All these are very important factors that complicate the use of this type of cells on a routine basis (Guillouzo et al., 2007; Gomez-Lechon et al., 2008; Guguen-Guillouzo and Guillouzo, 2010; Andersson et al., 2012).

1.5.3. HepaRG cells line

HepaRG cell lines are relatively new human cell lines which can differentiate to both hepatocyte-like and canaliculi-like cells. They are originally taken from a liver tumour of a human donor with chronic hepatitis C (Parent et al., 2004; Andersson et al., 2012; Anthérieu et al., 2012). HepaRG cell lines were reported to be the most comparable to primary human hepatocytes and human liver tissue among other liver cell lines (Hart et al., 2010)

HepaRG has many improvements over primary human hepatocytes in *in vitro* metabolic and toxicological studies. When compared to primary human hepatocytes, HepaRG cells lines have unlimited life span and are easier to handle, in addition to the stability of enzymatic phenotyping while primary human hepatocytes have variable enzymatic phenotyping. However, HepaRG cells

lines have some limitations mainly as they are of a particular genotype which affects the type of produced drug-metabolizing enzymes. HepaRG cells lines mainly produce intermediately active CYP2D6 and CYP2C9, while other genotypes such as CYP1A2, CYP2A6 and even CYP2D6 (one on the main produced CYP) are considerably less expressed in HepaRG cells than in primary human hepatocytes. This in turn leads to reduction in their metabolic capacity (Andersson et al., 2012).

In general, different strategies have been proposed to generate metabolically competent immortalized hepatocytes: transformation of human hepatocytes with plasmids encoding immortalizing genes, hepatocyte-like cells derived from stem cells, cell lines generated from transgenic animals and hepatocyte/hepatoma hybrid cells. Moreover, heterologous recombinant models expressing P450 enzymes in different host cells have been developed and successfully used in drug metabolism testing.

1.6. Cell death and cytotoxicity

Cell death is usually described as a process rather than an event, though there is no clear definition of cell death in literature. For that, cells are described as 'dying' in a reversible course until passing the point of irreversibility or 'point-of-no-return'. However, even this 'point-of-no-return' in the literature is not clearly defined, as the leading events to this are not clearly defined. Since 2005 and subsequently on 2009, the Nomenclature Committee on Cell Death (NCCD) is suggestively updating the criteria and recommendation to unify the definition of cell death within the scientific community. They classified cell death depending on four major criteria: morphological, enzymological, functional and immunological criteria (Kroemer et al., 2008; Galluzzi et al., 2011).

Cytotoxicity describes the effects that chemicals have on cells, which usually ends in cell death, though some toxic effects are reversible causing disruption of the function of the cell. Cytotoxicity studies are a very important part of any study on drug effects on cells and very commonly used as screening tools. It has much application, not only in the toxicology field, but also in other important fields, especially in the drug discovery and the drug industry. A common approach is the use of exclusion tests that involve the evaluation of the integrity of the cell membrane as a measure of cell

viability and cytotoxic effect. Another common approach is the use of colorimetric reaction that involves monitoring the chemical capacity of the cell to reduce specific agents. These types of tests have the advantage of being easy to implement and time-effective, where variable compounds at variable concentrations can be tested competently at the same time.

Exclusion tests depend mainly on the integrity of the cell membrane and its ability to keep impermeable to dyes, where damaged cell membrane will allow the dye to pass through and stain intracellular structure. Intact cell membranes will protect the intracellular environment and exclude the dye, keeping the cell unstained. The procedure simply consists of mixing the dye with cell suspension and then examining whether cells take it up (dead cells with damaged cell membrane) or exclude the dye (viable cells with intact cell membrane). (Longo-Sorbello et al., 2005).

Trypan blue, naphthalene black, propidium iodide and eosin are some examples of the vital dyes used in exclusion tests. Trypan blue is one of the most commonly used vital dyes in exclusion tests, a simple and non-expensive alternative. Trypan blue will stain dead cells cytoplasm dark blue while keeping viable cells clear with surrounding refractive ring (Longo-Sorbello et al., 2005).

Colorimetric assays are useful techniques to estimate viable non-damaged cells using microplate reading spectrophotometer. The cleavage reaction of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) salts to form the blue coloured non-soluble product (formazan), is one of the most commonly used procedures. This reaction take place only in viable cells by one of the mitochondrial enzymes, and the amount of produced formazan is directly proportional to the number of viable cells, which make it a very useful tool to study quantitatively the viable percentage of cell population (Vignati et al., 2005; Longo-Sorbello et al., 2005).

1.7. Sample preparation

Separating the analytes of interest from interfering materials, especially when biological samples are used, is one of the major challenges met while developing analytical methods. In the case of the analysis of drugs and their metabolites in biological fluids, where they are commonly existing in low

concentrations with the presence of large number of biological materials, sample preparation is most essential (Ahuja and Diehl, 2006; Snow, 2007).

The need for many steps of sample preparation makes it one of the most time consuming steps of analytical method development. Automated systems have been applied for sample preparation especially in laboratories overloaded with repetitive analyses. However, these systems are not popular as they are unreliable, inflexible and they need to be continuously maintained (Biddlecombe and Smith, 2004; Sastre and Szymanowski, 2005; Ahuja and Diehl, 2006). The primary aims of sample preparation are: producing representative sample of the original; isolation of analytes of interest from other unwanted interferences; pre-concentration of the analytes of interest; introducing the analytes onto the analytical instrumentation in a suitable solvent/carrier; and exclusion of biomaterials that may lead to analytical system decay, either physically or chemically.

1.7.1. Sampling

Sampling involves sample assortment, carrying, storage and later, selection of the sample before introducing in a suitable form into the analytical system. The choice of sampling techniques depends merely on the nature of the matrix, and the chosen type and method of sampling will affect the quality of the results, which in turn would affect data interpretation.

Sampling should assure minimal loss, degradation, instability, contamination or any other external interference with the sample. Sample collection and latter sample selection should be representative of the original sample, which raises the issue of homogeneity of the original collected and selected sample. Other negative effects on the quality of sampling by other factors (i.e. transport and storage) may be minimized by using preservative techniques. Commonly used techniques for preservation of the sample are proper choice of sampling container, addition of chemical stabilizers and freezing the sample. (Biddlecombe and Smith, 2004; Ahuja and Diehl, 2006).

1.7.2. General approach for sample preparation of different types of samples

After proper sample collection, transportation and sample selection comes the step of sample preparation. Sample preparation methods are usually aiming to clean the sample from interferences, and to separate the sample into its subcomponents and extract the analytes of interest.

Biological samples, the primary matrices dealt with in forensic analysis, are very complex matrices as they contain many components that may affect the quality of separation and analytical signal. Whatever the type and nature of the biological sample, a proper preparation is an inevitable prerequisite for introducing the sample onto the analytical instrumentation or performing other analytical steps (Biddlecombe and Smith, 2004; Ahuja and Diehl, 2006).

Biological samples need usually different sample preparation techniques when compared to other types of samples. This is very important to preserve the integrity and activity of the biological sample. Either urine samples may need pre-treatment by only adjusting the pH before extraction, using strong acids or strong bases to ensure basic analytes or acid analytes, respectively, are freely solvated in the sample. Serum and plasma samples most of the time need to be pre-treated in the same way as urine before extraction, mainly to free the protein-bound analytes. Liquid samples are easier to prepare when compared to solid or volatile samples, as liquid samples may not be in need of extraction or dissolution before introduction onto the analytical system. However, liquid samples usually contain many more interfering components and, relatively, low concentration of the analytes of interest. Solid samples may need more complex preparation procedures, depending on whether the whole sample or only part of it is of interest.

The two-phase system is the most common approach in sample extraction, where in this system the analytes of interest are distributed into one phase while the interfering components are distributed into the other. In liquid-liquid extraction, the distribution is into two immiscible liquid phases, while in liquid-solid extraction the distribution is into one liquid and another solid phase (Biddlecombe and Smith, 2004; Ahuja and Diehl, 2006).

1.7.3. Sample pre-treatment of different types of samples

1.7.3.1. Solid samples

Solid samples preparation may need only dissolving the whole sample in a suitable solvent, which is more applicable to homogeneous samples such as drug tablet formulation. Prior to that, physical techniques (e.g. grinding) maybe enough to turn solid a sample into smaller sized particles, easing dissolution of the sample and increasing the efficiency of further extraction steps. Sonicating the sample after will increase the efficiency of the whole process, as the ultrasonic action and the heat produced will ease the dissolution of the ground particles that will enhance sample recovery. Solid samples with water content, like soil, may need to be evaluated for water content before analysis to interpret final results properly, as water content will definitely affect the results. Drying of the sample and weighing it before and after will usually be enough to evaluate the relative amount of water and adjust results accordingly.

Generally, most solid samples are pre-treated by a solid-liquid extraction technique. The basic steps of this technique include physical removal of remaining insoluble interference (e.g. centrifugation) after homogenizing the sample with a buffer or an organic solvent. When an aqueous solvent is used to dissolve the solid sample, the resultant solution may be further extracted into an organic solvent by solution-solvent extraction, which is similar to liquid-liquid extraction (Wells, 2003; Biddlecombe and Smith, 2004; Ahuja and Diehl, 2006).

1.7.3.2. Volatile samples

Volatile samples are pre-treated by a different technique; usually in preparation for gas chromatography, the one tool commonly used for the analysis of volatile samples. The use of one or more techniques depends on the laboratory setup, researcher experience, analytical needs and physicochemical properties of the sample.

In headspace sampling, the sample is placed in glass vial having enough space above the sample. The glass vial is then heated and the above space will be occupied by the gaseous phase of the volatile

analyte of interest. The volatile analytes are then allowed to reach equilibrium with the whole sample, and the gaseous phase is then sampled and analysed.

In solid phase trapping, the gaseous sample is passed in a tube through a solid support, the captured vapour analytes are then extracted into a suitable solvent, and the solvent – with the extracted analytes - is analysed. Compared to solid phase trapping, in liquid phase trapping, the gaseous sample is passed through a liquid and the liquid, with the captured analytes; is analysed. In comparison, in liquid trapping, the gaseous sample is passed into an extraction solvent with affinity to the analytes of interest that is later analysed either directly or after being processed with one or more preparation steps.

The 'Purge and Trap' technique is relatively similar to headspace sampling. The sample is similarly heated and the vapour is allowed to collect in the space above the sample. However, in this type of technique, the vapour analytes are washed continuously, and the collected vapours are either directly analysed or go into further preparation steps.

In grab sampling, the gaseous sample is simply collected in a container and then rinsed with a solvent that is injected onto the analytical system, either directly or after pre-treatment. The sample can be collected simply using a bag or in a more advanced way using instrumental apparatus (Peach and Carr, 1986; Ahuja and Diehl, 2006)

1.7.3.3. Liquid samples

Liquid samples are the most common type of samples to be faced, and sometimes need only dilution before being introduced onto the analytical system. Liquid samples are commonly the product of one or more sample preparation steps of other types of samples as discussed earlier, but can be genuinely liquid.

In filtration, the simplest technique to pre-treat liquid samples, the liquid is passed through filter paper. This procedure could be enough to remove most of the solid interferences. In comparison, in microdialysis technique, the analytes are allowed to move from one liquid to another through a

membrane placed in between these two liquids. This is very useful technique to remove large sized protein from a sample prior to liquid chromatography. Centrifugation is another commonly useful technique to pre-treat liquid samples, and will remove efficiently most of the suspended solid interferences from the sample. Liquid-liquid extraction and solid phase extraction are of the most common techniques used for liquid sample preparation (Ahuja and Diehl, 2006).

1.7.4. General approaches for sample extraction

1.7.4.1. Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a technique based on the separation of analytes from a liquid sample matrix into another liquid. The two liquids are immiscible and typically, one of them is of aqueous nature while the other is an organic solvent. Typically, as most of the drugs are lipophilic or of organic nature, the analytes distribute between two immiscible liquids with preferable distribution into the organic phase, leaving behind the interferences in the aqueous liquid (Wells, 2003; Sastre and Szymanowski, 2005; Ahuja and Diehl, 2006).

Because of its advantages, LLE is still of the handiest techniques for sample preparation. LLE is a simple, relatively inexpensive and with high separation capacity. However, it is disadvantageous because of the high volumes of solvents needed, not being applicable to all types of analytes, emulsion formation and the difficulty to automate the process (Wells, 2003; Sastre and Szymanowski, 2005).

The ratio of partitioning of the analytes between the two immiscible liquids depends on many factors related mainly to the properties of the analytes and the liquids. The analytes will preferably partition onto the organic solvent when they are in their unionized form, and when they show solubility in the organic solvent. The selection of the extraction solvent would affect the ratio of partitioning and improve the efficiency of extraction process. (Wells, 2003; Ahuja and Diehl, 2006).

An ideal extraction solvent is the one that is insoluble in water, of low viscosity, of low boiling point, of high affinity for analytes and has the lowest hazards. The low viscosity will allow the solvent to mix easily with the biological sample – the very common type of samples in forensic settings, while

the low boiling point will make it easier to evaporate the solvent – a very common step after extraction. Changing pH values of the matrix liquid and ratio of organic solvent to aqueous liquid ratio, in addition to the type of solvent, are other important factors adjusting the extraction process. Changing pH values affect the ionization state of the analytes, controlling how these analytes partition between the immiscible liquids (Ahuja and Diehl, 2006). According to the distribution law that was derived by Nernst in 1898, unionized analytes will distribute between two immiscible liquids at a constant rate. In typical settings, though in practical settings the assumption of the presence of ideal solvent is invalid, Equation 1 applies at equilibrium

$$\boldsymbol{K_D = \frac{[X]_{org}}{[X]_{aqu}} mv^2} \qquad \text{Equation 1}$$

Where K_D: Distribution constant, $[X]_{org}$: concentration of analyte X in organic liquid and $[X]_{aqu}$: concentration of analyte X in aqueous liquid

Applying (Equation 1), a high KD value indicates high ratio of partitioning in favour of the organic phase, which reflects an efficient extraction process. Many parameters can be adjusted to increase the KD value. For example, Adjusting the pH value to make the analyte uncharged, selection of a solvent where the analytes preferably partition into and using high volume of the extraction solvent, all would increase the KD value and hence the amount of the extracted analytes (Ahuja and Diehl, 2006; Dey, 2014).

From a practical point of view, adjusting the pH would allow the separation of different types of analytes into neutral, basic and acid analytes. The best results of extraction are achieved when adjusting the pH values two units below the expected pKa value in the case of acidic analytes, while adjusting it two units above the expected pKa values in the case of basic analytes (Wells, 2003; Biddlecombe and Smith, 2004). Increasing the surface area of contact between the immiscible liquids is another important factor that would speed the equilibrium process. A large surface area of contact between the two liquids would ensure rapid equilibrium, and this can be achieved by thoroughly mixing the two liquids, either mechanically, manually or using vortex. Emulsion formation, one of the major disadvantages of LLE, would cause inefficient extraction of the analytes, as they will be

trapped within the emulsion. Gentle mixing of the liquids and using large volumes of the extraction solvent may help to reduce the formation of emulsion (Biddlecombe and Smith, 2004). LLE provides efficient sample clean-up and concentration, and evaporating the resultant organic fraction containing the extracted analytes followed by reconstitution with compatible solvent will produce a sample ready for analysis.

1.7.4.2. Solid phase extraction

Solid-phase extraction (SPE), as a method of sample preparation, is a classical method, but still one of the techniques commonly used for separation of drugs and metabolites from complex biological matrices in preparation for chromatographic analysis (Wells, 2003; Ahuja and Diehl, 2006; Snow, 2007). The history of SPE dates back to the1950s, and commercial availability to the 1970s. Since then, SPE has become one of the most popular sample preparation methods (Snow, 2007). The basic principle of SPE is to adsorb the analytes onto a surface and then wash the surface with elution solvent, either keeping the interferences behind in the original solution after adsorption of the selected analytes, or adsorbed onto the surface after washing with elution solvent. Due to the described principle of extraction, SPE is more commonly termed sorbent extraction. A classical SPE extraction system consists of sorbent particles packed into a cartridge, the sorbent consists of porous particles of chemically modified silica backbone and the packing is held in place using frits. The silica backbone is modified by binding different types of organic moieties. The different type of moieties that can be bound to the silica backbone will afford a countless number of application of SPE system. (Wells, 2003; Ahuja and Diehl, 2006; Snow, 2007).

A typical process of SPE mainly consists of four steps: conditioning, sample addition, washing and elution. In step 1, the sorbent is conditioned with one or more solvents, depending on the expected chemical properties of target analytes, where one of these condition solvents is the elution solvent. Conditioning is aiming to prepare the sorbent for the extraction process. This will guarantee the functional moieties, the ones attached to the silica backbone, are appropriately solvated, assuring the best exposure of the analytes to these functional moieties and hence the maximum extraction recovery. In step 2, the sample is applied onto the sorbent, drawn through the sorbent usually by

vacuum suction or pushed through with pressure applied by a plunger. The chosen type of sorbent should have high affinity for the analytes of interest. The amount of extracted analytes depends on the partition coefficient between the sample and the sorbent, similarly to the previously discussed liquid-liquid extraction (LLE). The sorbent in step 2 will retain the analytes and maybe some interfering materials adsorbed onto the surface matrix. In step 3, the sorbent is washed with one or more solvents that have the ability to release the adsorbed interferences yet retain the analytes. In the final step, step 4, the sorbent, with the analytes adsorbed onto its surface matrix, is eluted with a solvent that has the ability to release the adsorbed analytes only (Figure 6).

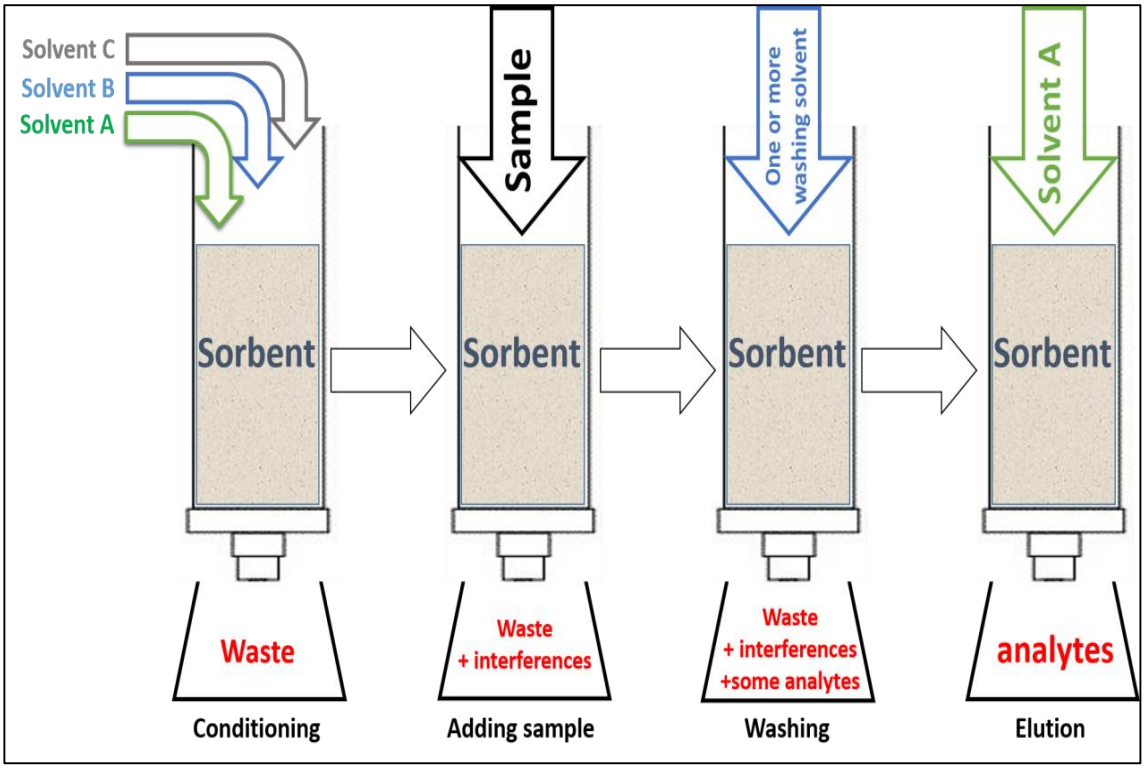


Figure 6: schematic representation of typical SPE protocol

The resultant extract is made ready for analysis in the same way as described for LLE during the extraction process. The pH adjustment of sample solution, washing solution and elution solvent plays a major role to increase the efficiency of extraction. (Wells, 2003; Ahuja and Diehl, 2006; Snow, 2007).

Compared to other sample preparation techniques, SPE appears to be superior and more advantageous. For example, compared to LLE, SPE uses significantly lower volumes of solvents,

has lower risk of exposure to hazards, has no risk of emulsion formation, has lower risk of contamination, is relatively inexpensive and generally has wider range of extraction applications (Ahuja and Diehl, 2006; Snow, 2007).

The type of the functional moiety bounded to the silica backbone is very wide and varied, and the choice of one type depends on the nature of analytes to be extracted. Hydrocarbons, anion or cation exchange functional groups can be bound to the silica backbone, depending on the type of application. The most common type of sorbent used in SPE is the one with a bound hydrocarbon functional moiety, most commonly C_{18}. This is similar to the most common type of sorbent used in chromatography columns. The hydrocarbon moiety provides a nonpolar and hydrophobic surface, the one preferable to adsorb the common type of analytes needing analysis in toxicology – drugs of abuse and their metabolites (Ahuja and Diehl, 2006; Snow, 2007).

1.8. Basic principles of Chromatography

In 1993, The International Union of Pure and Applied chemistry (IUPAC) presented a definition for all terms, symbols used in chromatography, and defined: 'Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction' (IUPAC, 1993).

The history of modern chromatography dates back to the start of the 20th century when the Russian scientist Twsett separated chloroplast pigments using $CaCO_3$ as a stationary solid phase and petroleum ether as a mobile phase, though the basic principles of separation were presented earlier (Grob and Barry, 2004; Little, 2008). Thereafter, there was tremendous developmental milestones in the field of chromatography, reaching the status of being a major technique in many science fields (Table 1)

Table 1: Major developmental milestones in the field of Chromatography

Scientist		Major Development
1900s	Twsett, M.	Separated chloroplast pigment
1940s	Tiselius, A.	Adsorption analyses. Liquid chromatography. Pointed out different modes of chromatography.
	Consden, R., et al.	Paper chromatography
	Claesson, S.	Liquid–solid chromatography
1950s	Cremer, E.	Gas–solid chromatography.
	Phillips, C. S. G.	Liquid–liquid chromatography (Frontal mode)
	James, A. T., and Martin, A. J. P	Gas–liquid chromatography
1960s	Giddings, J. C.	Reviewed theories of chromatography

As pointed out by Tiselius in 1941 with current application up to date, there are three modes of chromatography analysis: Frontal analysis mode, Displacement mode and Elution mode.

In frontal analysis mode chromatography, the sample mixture acts as its own carrier (i.e. mobile phase) when injected into the column that contains the stationary phase. The sample mixture components separate depending on the capability of each component to bind - and so be retained - by the stationary phase, based on their affinity. A variety of this technique is the zonal elution analysis, where the sample is injected in small volume rather than continuous flow (Grob and Barry, 2004; Calleri et al., 2012). The least binding component is the one eluted firstly in its pure form, and with the continuous flow elute the second least binding is eluted and so on. The term 'frontal analysis' relates to the initial use of this method for the assessment of the alteration in concentration of the front exit of the column. This method is disadvantageous for the need of large quantity of sample mixture, and for that it is used mainly as preparative chromatographic technique.

In displacement mode chromatography, a mobile phase, either liquid or gas, is used to carry the sample mixture. The mobile phase must have more binding capacity to the stationary phase than any

component of the sample mixture. This method has application in LC, or as a preparative step in analysing traces in GC (Grob and Barry, 2004).

In elution mode chromatography, the components in the sample mixture, carried by a mobile phase, move over the column at different rates according to their binding capacity on the solid stationary phase (Grob and Barry, 2004). In modern practice, nearly all methods of chromatography are performed in elution mode, to the extent all other modes are negligible other than as preparative steps (Little, 2008). Ideally, in this mode of chromatography, the components of the sample mixture would elute from the stationary phase in separated peaks on a baseline, achieving the best resolution. The travel speed of each component through the stationary phase depends mainly on the interaction between each component and the stationary phase. This interaction can be maximized to obtain the best resolution by adjusting the operation parameters (low column loading, gradient elution ... etc.). Elution mode has drawbacks of being time consuming and operationally complex (Grob and Barry, 2004; Little, 2008).

1.8.1. Liquid Chromatography

The HPLC technique is based on a solid (stationary) phase and a liquid (mobile) phase. This contrasts with Gas Chromatography (GC), where the technique is based on a gaseous (mobile) phase. HPLC is superior for the analysis of organic compounds, which are mostly non-volatile or unstable. HPLC is the core instrumentation used for analysis of drugs of abuse, where reversed phase chromatography (RPC) is the main utilized HPLC mode. It is a combination of a non-polar stationary phase (the column), and a polar mixture of water and an organic solvent-mobile phase (Mc Fadden et al., 2006; Snyder et al., 2009; Bayne and Carlin, 2010).

Liquid chromatography instrumentation consists of four major components: sample introduction, mobile phase, stationary phase and detector component.

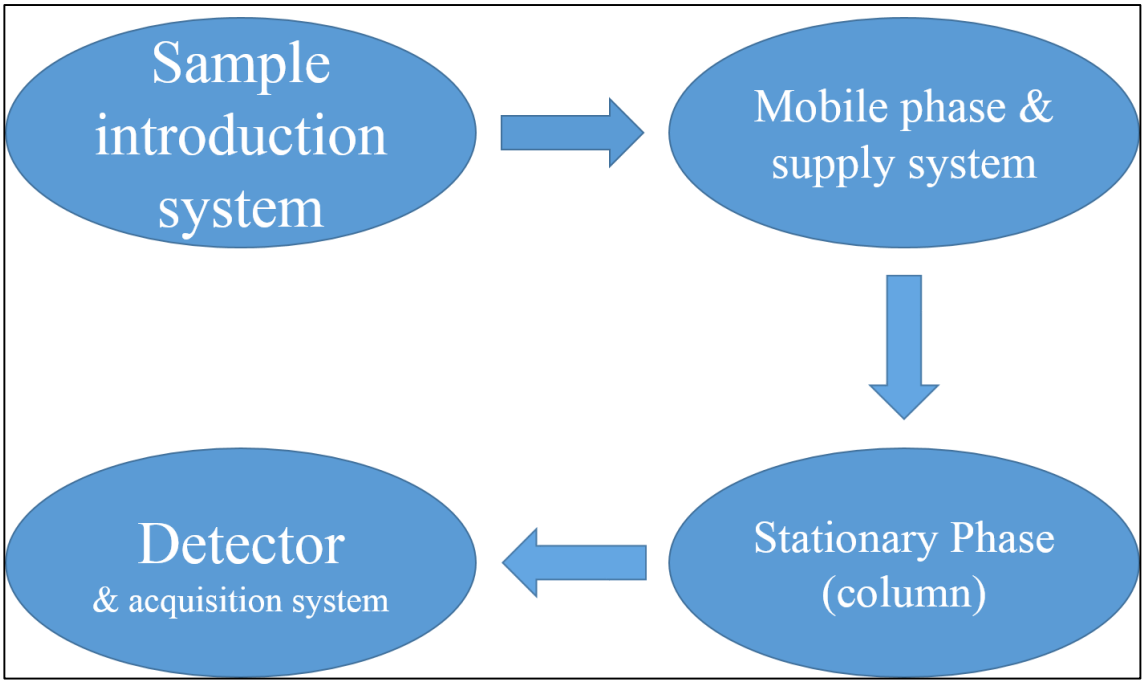


Figure 7: schematic representation of major components of liquid chromatography system

1.8.1.1. Mobile Phase

The mobile phase major component is the pump that should deliver stable flow rate depending on the type of mounted interface and diameter of the separation column. This stability in flow is more crucial when the LC is coupled into MS instrumentation. The type of the mobile phase is extremely important because, as has been discussed earlier, it is the interaction between the analytes, mobile phase and stationary phase that determines how these analytes separate.

Reversed phase chromatography mode is the most commonly used in most liquid chromatography applications. In this mode of chromatography, a relatively high polar mobile phase, in comparison to the polarity of the stationary phase is used for separation. Mobile phases are usually prepared as a mixture of solvents. Buffers are added to the mixture to control the pH and hence degree of ionization and provide reproducible peaks. The type and degree of buffering depends on the type of application and mounted interface.

The composition of mobile phase, i.e. type and ratio of solvent mixture, is another important factor when separating different types of analytes. For example, a mobile phase of specific composition that is efficient for the separation of polar analytes will not separate non-polar analytes with the same

efficiency, and vice versa. In the presence of different analytes of different polarities the change of the mobile composition as the analysis progress will separate different analytes with different polarity more efficiently. A separation using a mobile phase with the constant compositions is called 'isocratic elution' in contrast to 'gradient elution' where the composition of the mobile phase is changing with time (Ardrey, 2003; Snyder et al., 2009; Bayne and Carlin, 2010).

1.8.1.2. Sample introduction system (Injector)

The sample introduction and the injector component is usually a valve injector. The function of the injector is to introduce the sample in its liquid form onto the flowing mobile phase stream (Figure 8). The mechanism of introduction is quiet simple, but very important. When the rotating valve is in position A, a loop with specific volume is loaded by the sample solution while the mobile phase is continuously flowing onto the column. When the rotating valve switches to position B, mobile phase flow will be diverted through the loop in the opposite direction of the sample load, flushing the loop contents onto the column, (Ardrey, 2003).

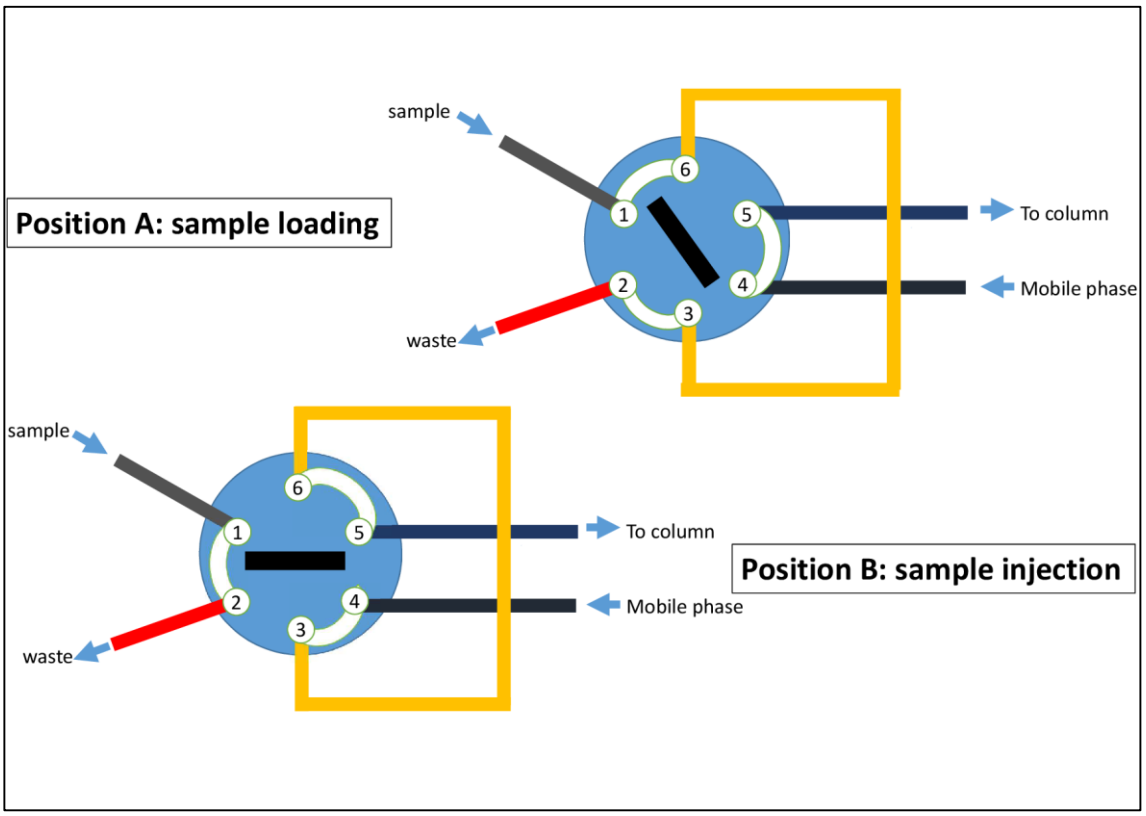


Figure 8: schematic diagram of sample introduction system and its mechanism of function

1.8.1.3. Stationary phase

The type of stationary phase, as discussed earlier, is another important factor that determines the efficiency of separation. It has been mentioned that reversed phase chromatography is the most commonly used mode in chromatography. For that, a low polar stationary phase, in comparison to the polarity of the mobile phase is the most common type of stationary phases used in chromatography. The most commonly used columns are those with a chemically modified silica backbone, mainly with C_{18} alkyl groups. As the column length increases, separation efficiency will also be increased. However, the working pressure and analysis time will also be increased. As the internal diameter of the column is squared, the amount of the sample that can be analysed increases. As the average packing particle diameter is squared the separation efficiency and working pressure decreases (Ardrey, 2003; Bayne and Carlin, 2010; McCalley, 2010).

1.8.1.4. Detectors

A number of detectors are in routine use, depending on the type and application of analysis, and the properties of the analytes, solutes and solvents used for analysis. UV-Vis detector is one of the most widely used type of detectors with liquid chromatography. Analytes with known wavelength of maximum absorption can be monitored selectively through this type of detector. However, with the availability of diode array detectors (DAD), it is more accurate to record the spectral scan of the absorbance at different wavelengths, which could be used more selectively for the identification of the analyte. HPLC instrumentation is commonly coupled to MS instrumentation as a detector, which is very advantageous for analysis of different compounds, both qualitatively and quantitatively (Ardrey, 2003).

1.8.2. Gas chromatography

Gas chromatography (GC) is widely applied in analytical chemistry and one of the main tools in forensic laboratories for separating and analysing compounds that are able to evaporate without being decomposed. Mobile phase in GC is typically a non-reactive carrier gas, such as helium or nitrogen, while the stationary phase is a solid or liquid. The major components of a GC instrument are: source of carrier gas, port of injection, column, detector and a recording device or PC. The sample mixture

components shall interact with both the stationary phase and the carrier gas. The separation of the sample mixture components depends on their interactions with the stationary phase and their vapour pressure. Temperature during the separation procedure in GC must be controlled slightly higher than the expected boiling point of sample mixture components. The choice of detectors in GC depends on the physicochemical properties of the tested components. Common detectors are thermal conductivity, flame ionization and mass spectrometry detectors. Compared to the time consuming LC, the time of analysis is generally shorter in GC. Analysis time can be reduced by using shorter and narrower columns, higher temperatures and faster flow rate. However, the use of high temperatures may worsen the resolution (USP; Hinshaw, et al. 2013, Grob, 2004).

1.9. Basic principles of Mass Spectrometry (MS)

Mass spectrometry is one of the fundamental analytical tools in chemistry, biochemistry, pharmacy, and medicine. It is also one of the essential tools in toxicological laboratories. Mass spectrometry was introduced as a routine technique in the 1950s, providing the molecular formula for compounds and a closer insight into structure and fragmentation patterns.

The basic principle of mass spectrometry is based the motion of charged ions in an electric or magnetic field, which is achieved by creating gas phase ions from molecules and then separating these ions according to their mass-to-charge ratio (m/z) and finally measuring the abundance of each ionized fragments by the detector. Mass spectrometry can be used for both qualitative and quantitative studies. It can provide data about the mass and structure of the atoms or molecules, which can characterize and identify the material of interest.

The mass spectrometer separates gas phase ionized fragments and atoms based on the difference in (m/z), where (m) is the unified mass unit which equals one Dalton (Da), and (z) is the charge on the ion, either positive or negative. As the ionized fragments travel and are separated in the spectrometer, a spectrum is produced graphically. This spectrum represents a plot of m/z values against the relative abundance. The produced spectrum shows fragmentation patterns of the analyte which, in combination with m/z values, are used to determine the molecular weight and structure of the analyte.

A mass spectrometer is typically composed of five main components: sample input system, ionization source, the mass analyser, detector and computer-based data processing system (Figure 9).

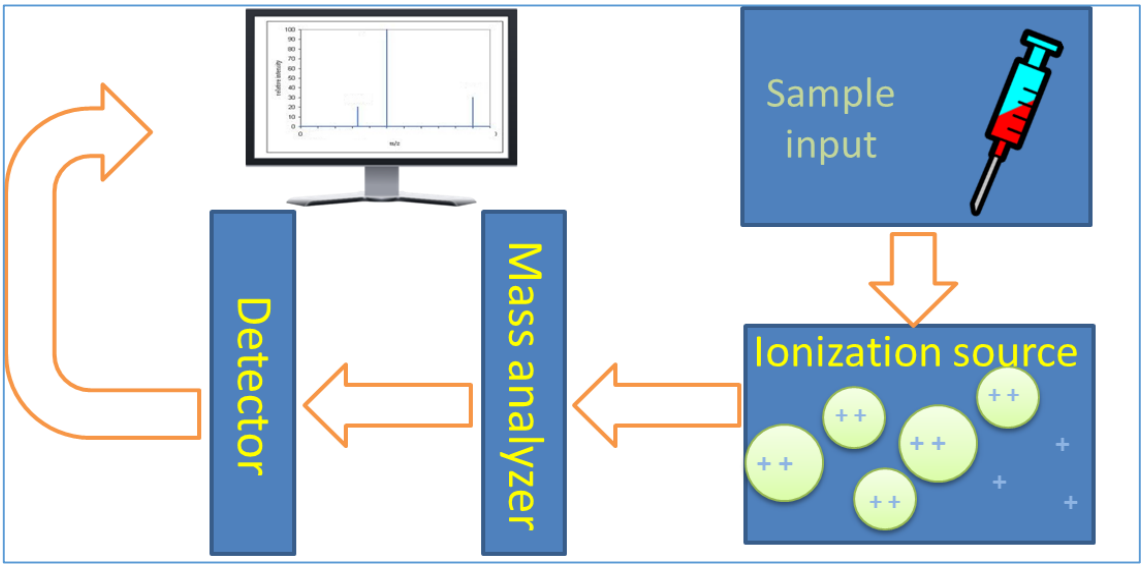


Figure 9: Major components of Mass Spectrometer.

1.9.1. Sample input system

There are different methods for the input of the sample onto the mass spectrometry system; each with its application with advantages and disadvantages. The selection of the sample input system depends upon the nature of the sample, sample matrix, and sample physicochemical properties. As to be discussed later, ionization techniques are typically designed for gas phase molecules, which needs the sample to be introduced onto the mass spectrometry system in gas phase. The most basic sample input method is directly injecting the sample in vaporous gas phase onto the ionization source of the MS system. This method is called direct infusion. Where the analyte itself, or dissolved in a solvent can be directly injected onto the ionization source component of MS system. This method is applicable for liquids with low boiling point and high risk of contamination.

Currently, chromatography instruments are the most commonly used sample front end, either: gas chromatography (GC) or liquid chromatography (LC). This allows the separation of a mixture sample before being introduced onto the ionization source of mass spectrometry system.

1.9.2. Ionization source

The mass spectrometer detects molecules by using charged molecules (ions), which mandate the molecules to be charged or ionized in advance before introduction onto the mass spectrometer. In addition, as the process takes place in a vacuum, those ions must be introduced in the gaseous phase. The process of ionization and production of the gaseous phase is easily done for volatile samples, but it becomes more challenging for thermally labile molecules. The choice of the ionization method depends mainly on the nature of the sample, but also on the type of information required. There are two classical methods for ionization: electron ionization (EI) and chemical ionization (CI), with different applications.

The principle of EI is to pass a beam of electrons through a gas phase sample producing, most of the time, single charged molecular ions, but also negatively charged molecular ions can be produced. In addition to ionizing the target molecule, EI would fragment the molecule into smaller fragments producing single charged smaller fragment ions. This method is referred in the literature as hard ionization source due to the high energy used, and much fragmentation is produced a consequence. This much fragmentation is very useful for structural elucidation of unknown spectra. EI is the most common ionization method used for MS, and it is best applicable to volatile substances.

The principle of CI is to introduce a large excess of reagent gas relative to the sample, into the ionization region allowing the electrons to bombard the mixture. As reagent gas is present in excess, it is more likely to be ionized than the sample itself, which allow the sample to be ionized by collision with gas molecules. This method is usually referred in the literature as soft ionization source, and it causes less fragmentation but more molecular ions.

Fast Atom Bombardment (FAB) and Secondary Ion Mass Spectrometry (SIMS) are another two ionization methods. In both techniques, high impact energy, without the need of heat, is used. This

makes both these techniques more suitable for the study of thermally labile compounds. Atmospheric Pressure Ionization (API) sources ionize the sample at atmospheric pressure before being introduced onto the mass spectrometer, and they are applicable to ionize thermally labile samples.

Electrospray Ionization (ESI) is the most common ionization method for non-volatile thermal-labile molecules and high molecular weight compounds, and it can be easily coupled to liquid chromatography. ESI has many advantages: sensitivity, robustness and simplicity, in addition to the ability to couple continuous flow methods – like liquid chromatography - with MS (Van Bramer, 1997; Pramanik et al., 2002; Glish and Vachet, 2003; Ardrey, 2003; Gross, 2004; Herbert and Johnstone, 2010)

1.9.3. Mass analyser

As has been discussed earlier, the mass spectrum is mainly a plot of the number of ions for each m/z ratio produced by an analyte after been ionized. Generally, single ionized species are produced, so the spectrometer detects the mass (m value). The ions are streamed then into the mass analyser through series of electro-magnetic fields, and the stream of ions will be affected and deflected from their original trajectory path. The extent of deflection is affected by the ion's mass and type and the strength and geometry of the electro-magnetic fields.

When the ion passes through the electromagnetic fields, many forces will affect the direction and magnitude of deflection. The ions entering the electro-magnetic field are having a kinetic energy when accelerated out of the ion source, and for the practically single charged ions, equation 2 applies (Equation 2). Centrifugal force will act on the moving ion deflecting its path, depending on mass and velocity of the charged ions when entering the field. In case of magnetic field, the radius of deflection depends also on the magnetic field strength (Equation 3), and in case of electrostatic field, the radius of deflection depends on the electric potential (Equation 4)

$$\boldsymbol{V}=\frac{1}{2}mv^2 \qquad \text{Equation 2}$$

$$B = \frac{mv}{r}$$ Equation 3

$$E = \frac{mv2}{r}$$ Equation 4

Where:

V: acceleration potential applied to ion in the magnetic field.

B: magnetic field strength.

E: electric potential applied to ion in the magnetic field

m: mass of ion.

v: velocity of ion entering the field.

r: radius of ion deflection in the field.

For magnetic fields, rearranging the variables of (Equation 2) and (Equation 3) for velocity (v) and combining them for radius of deflection (r) then (Equation 5) applies

$$\boldsymbol{r=\frac{\sqrt{2mV}}{B}}$$ **Equation 5**

For electrostatic fields, rearranging the variables of (Equation 2) and (Equation 4) for velocity (v) and combining them for radius of deflection (r) then (Equation 6) applies

$$\boldsymbol{r=\frac{\sqrt{2V}}{E}}$$ **Equation 6**

Considering Equation 5 for magnetic field, the radius of deflection (r) is directly proportional to the mass of the ion (m) and acceleration potential of the filed, while inversely proportional to magnetic field strength (B), and in case both acceleration potential (V) and magnetic field strength are kept

constant, then radius of deflection (r) will increase with increasing ion mass (m), separating ions on different position according to their masses (Figure 10).

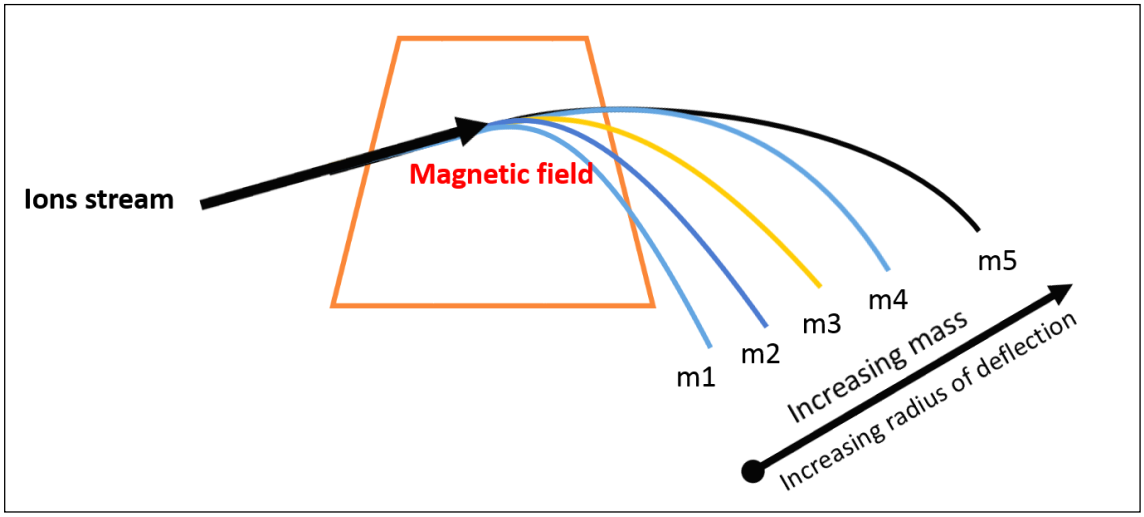


Figure 10 : Magnetic sector mass analyzer.

However, the modern instrumentation mandates to keep the radius of deflection to be constant to guide ions into a single position for collection and analysis. This constant radius is achieved by varying the acceleration potential (V) and magnetic field (B) strength. Rearranging the variables of (Equation 5) for ion mass (m) while deflection radius (r) is constant then (Equation 7)

$$m = \frac{B^2}{2V} \qquad \textbf{Equation 7}$$

It can be concluded from (Equation 7) that a single mass ion can be selectively monitored adjusting the acceleration potential (V) or magnetic field strength (B) or both variables.

Considering (Equation 6) for electrostatic field, the radius of deflection is dependent on acceleration potential (V) and electrostatic field strength (E) – i.e. acceleration potential of ion on entering the field and electrical potential applied on the ion in the field. Thus, ions having same kinetic energy will be selected and guided to the collection point, though may have different ion mass (Herbert and Johnstone, 2010)

Electromagnetic and electrostatic fields are both used in modern instrumentation analyses, in combination, sequential or crossed geometry. The aim is to focus adjust the ion stream to arrive the collection and analysis point in focus, according to their mass, kinetic energy or both. The stream of ions collector produces the mass spectrum, which is a measure of the different m/z values and their respective abundance.

Many mass analysers are available in use currently each with its advantage and disadvantages. The power of the mass analyser is measured by resolution (R), which is calculated mathematically as:

$$\mathbf{R=m/\Delta m}$$

Where R is the resolution, m is the *m/z* to be measured, Δm is the difference between *m/z* value, and the ion from which is to be separated.

The linear quadrupole ion trap mass analyser, 3D quadrupole ion-trap mass analyser, time-of-flight mass analyser, double focusing and trisector mass analysers are all different examples of currently used mass analyser.

The linear quadrupole ion trap mass analyser (quadrupole mass analyser or transmission quadrupole as termed in different literature) consists of four rods arranged geometrically parallel to each other, and each two opposite rods are connected to an electric current and voltage (Figure 11). On a quadrupole mass analyser, one opposed pair is connected to positive potential while the other opposed pair is connected to negative potential. The potential is of two components: fixed potential (U) and radiofrequency (RF). RF is of amplitude (V) and angular frequency (ω) represented by the function Vcos(ωt) and as cos(ωt) cycle with time (t), voltage (V) change in a manner resulting in net electric field of zero at any time and along the planes and axis of the quadrupole analyser. The motion of ions within the quadrupole field is quite complex when compared to the ones in electromagnetic and electrostatic type of fields. Adjusting the voltage will change the field strengths, will make a specific m/z value ion to oscillate in the central axis and on the two transverse plans, and to follow a stable path until reaching the detector, while others m/z values ions will be deflected to periphery toward the rods. Because the net electric field is zero, ions matching the selected m/z will trap in the

analyser and will not pass to the detector unless given an initial momentum. This momentum is provided through potential between the analyser and ion source.

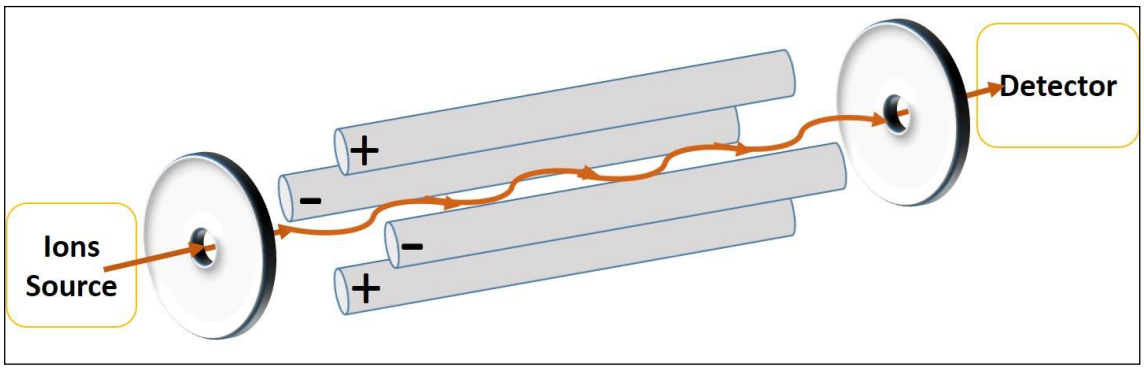


Figure 11: Linear quadrupole mass analyzer

The 3D quadrupole Ion-Trap Mass Analyser (ion trap or quadrupole ion trap as termed in different literature), as the name implies, is related to the linear quadrupole mass analyser. Both types of detectors were invented and developed by Paul and Steinwedel in the 1960 and this work was recognised by the award of Nobel prize in physics in 1989. Both types of detectors, linear and 3D quadrupole mass analyser are working in a relatively similar way, but the path through the 3D quadrupole mass analyser is more complex than that in linear quadrupole mass analyser. Both types, linear and 3D quadruple mass analyser are low-resolution devices and this maybe a disadvantage when studying the elemental composition (Stafford, 2002).

Time of Flight Mass Analyser (ToF) is the simplest mass analyser device (Figure 12). Working theory behind ToF instrument is applying the same energy on the ions while passing through, which will make each ion travel at a velocity inversely proportional to the mass, producing a spectrum related to the time each ion needed to reach the detector. This theory is basically different for other mass analysers, where there is no selection of specific mass in advance before entering the mass analyser, only one ion at a time enter the mass analyser. The first generation of ToF mass analyser were low resolution devices, but modification of the next generation improved tremendously the resolution of ToF. As the basic theory behind the work of ToF depends on the travelling speed and time needed for ions to reach the detector, increasing the length of the ToF tube would emphasise small difference on traveling speed of ions and hence the time needed to reach the detector. However,

this increase in the length of the tube is practically inappropriate as it would increase the size of the instrumentation The use of ion mirrors within the ToF tube increased the travelling distance without the need to increase the length of the tube which dramatically increased the resolution of ToF mass analyser thousands folds. In addition to the simplicity and fast scanning capability, ToF are increasingly used when high resolution analysis is needed. (Van Bramer, 1997; March, 2000; Pramanik et al., 2002; Glish and Vachet, 2003; Ardrey, 2003; Gross, 2004; Herbert and Johnstone, 2010).

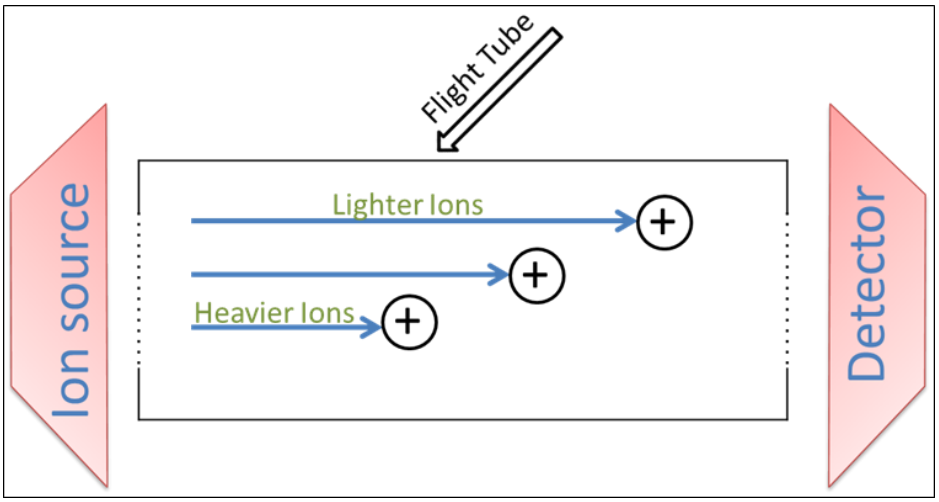


Figure 12: simple schematic representation of Time of Flight Mass Analyzer.

1.9.4. Ion detectors

In all types of mass spectrometry instrumentation, mass analysers would separate ions according to their m/z values to reach the detector area for the mass value and ion abundance to be detected and recorded. A mass spectrometry, as mentioned earlier, is a measure of the different m/z values and their respective abundance.

The ions, after being analysed, may reach selectively to the detector in a sequential pattern according to m/z value – i.e. one selected mass at a time. This mode of detection, sequential detection of one mass at a time, is referred to as 'point ion detector'. Other types of mass analysers' separate ions according m/z value but without focusing one m/z value, and ion stream reach the detector separated but dispersed. This mode of detection is referred to as 'array detectors'. In practice, mass spectrometry instrumentation may use one type or both of ion detectors.

1.9.4.1. Point ion detectors

Point ion detectors are the most in use for the common type of mass analysers. Three main types of point detectors are used: Faraday Cup, Electron Multiplier and Scintillator. Those three types of detectors utilize the phenomena of 'secondary electron emission', which refers to the emission of secondary electrons when ions or more widely charged particles strike a metal surface (Figure 13).

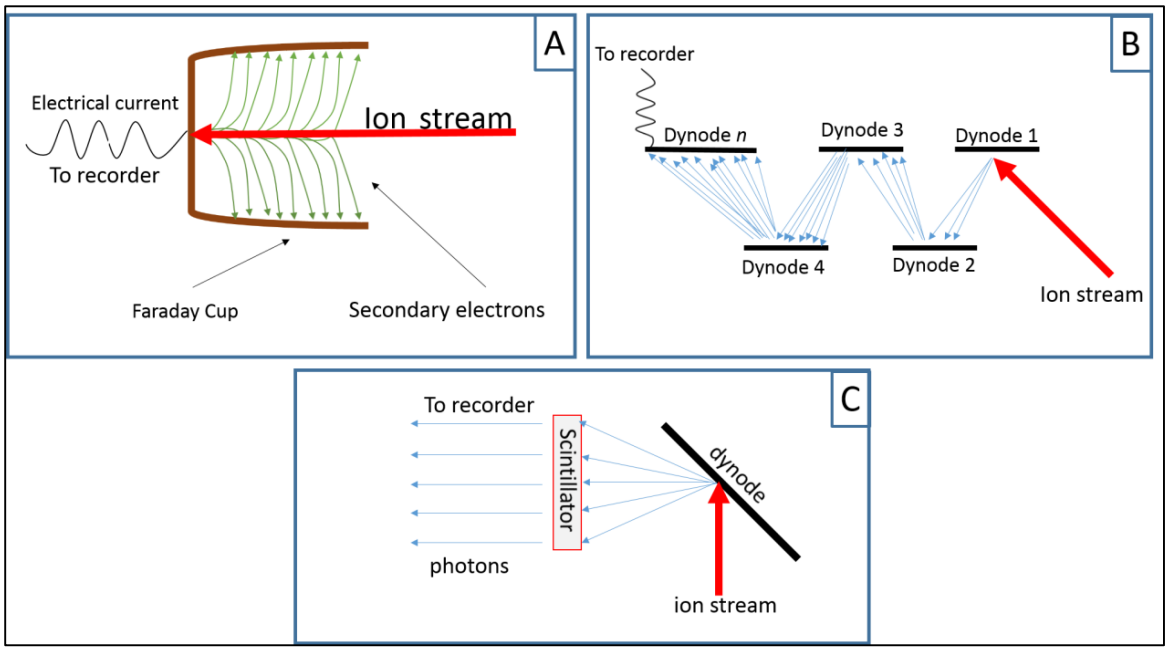


Figure 13: Schematic representation of different types of point ion detectors.
A: Faraday cup, B; electron multiplier and C: scintillator

In Faraday Cup ion detector, ions strike a metallic cup shaped collector and the secondary emitted electrons will be collected within the cup. The flow of electrons converts into an electrical current that is recorded respectively. In Electron Multiplier, the secondary emitted electrons when ions strike one plate (dynode) are accelerated to another plate and then to another third or more dynodes in serial. This will provide logarithmic multiplication of the ions, and the currently available electron multipliers have ten or more dynodes in serial providing millions times amplification power of the primary electron count. Again, the flow of electrons converts into an electrical current that is recorded respectively. In scintillator, or Daly detector, after the primary ion stream strike one dynode, the secondary electrons are accelerated to another dynode. In Daly detector, the second dynode is a scintillator that emits photons when stroke by electrons. In these types of detectors, the emitted

photons are converted into an electrical current that is recorded respectively (Herbert and Johnstone, 2010).

1.9.4.2. Array detectors

Mass analysers separate ions according to their m/z values, and either they focus the separated ions into one point or deliver them to the detector side, separated but dispersed in space. A typical example of the latter is magnetic sector mass analyser. The dispersed ions can be recorded simultaneously through a detector type consisting of number of ion collection elements arranged in a line (Figure 14).

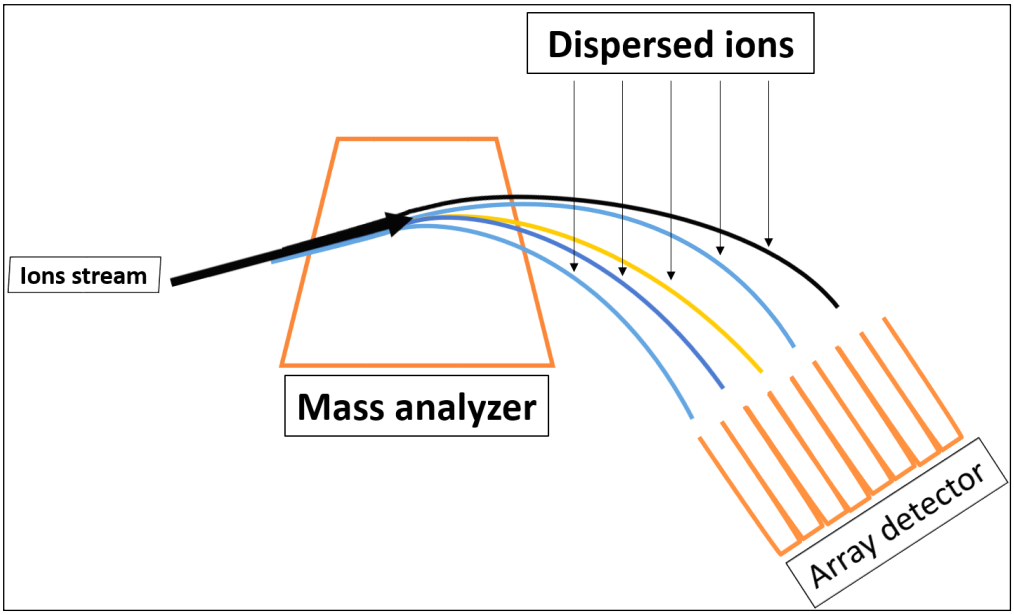


Figure 14: multichannel array detector

Single-unit electron collector is one-collection element represents an electron multiplier, utilizing the phenomena of 'secondary electron emission'. Compared to the 'one-point detector' type described earlier m single unit electron collector multiplier re much smaller in size, and the 'secondary electrons' emit when ions strike serially the side of the collection element, instead of serial dynodes, accelerated by potential (Figure 15).

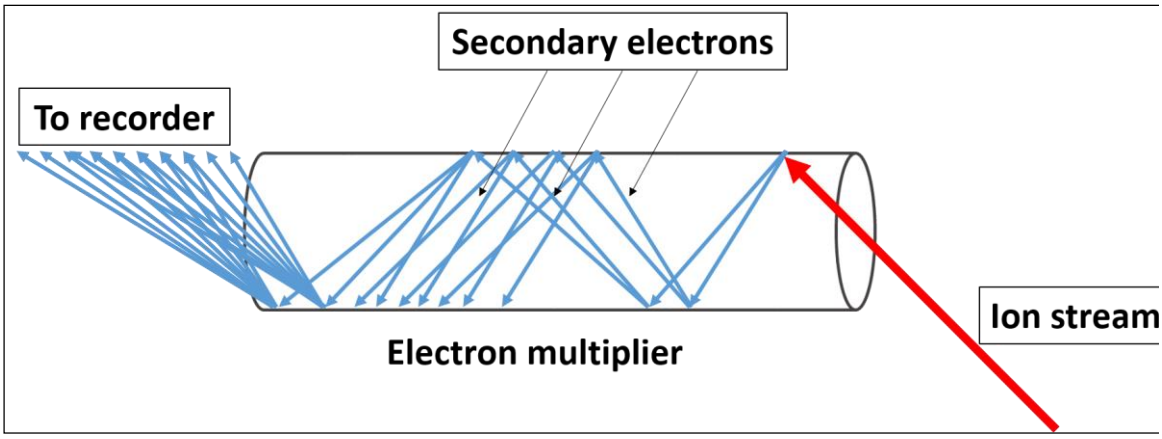


Figure 15: single unit electron collector

Due to the difficulty of fitting large number of 'collection elements' into an array, the range of ions can be scanned is narrow. For example, a compact array of 50 'collection elements' allow only a range of 50 m/z scan with integer values. However, assuming the need to increase the resolution to 0.5 instead of integer values, this allows only a range of 25 m/z scan. Hence, 'array detectors' allow wide range m/z scan with low resolution, or narrow range m/z scan with high resolution (Herbert and Johnstone, 2010).

Chapter 2 Literature Review

2.1. Current role of HPLC-DAD for the analysis of mephedrone and other selected NPS

At the start of this thesis in 2009- 2010, mephedrone was starting to appear and traded as legal high. Mephedrone was selected due to its high significance and succeeding reports about its toxic and morbid effects. At that point, no data were available about its analytical and toxicological profile apart from subjective data presented by mephedrone users in community discussion forums.

Though mephedrone was banned in most European countries in 2010, some fatal cases were reported after, and mephedrone has been traded in the illicit drug market. In addition, no reference standards were available which challenges researchers when developing analytical techniques for the detection of mephedrone, potential metabolites and toxic effects. This lack of data prompted the study of mephedrone, with other structurally related cathinones, with adulterants and other drugs of abuse. HPLC-DAD was the instrument of choice for preliminary studies about mephedrone because of its availability in most forensic laboratories and relatively low cost.

What follow is a literature survey about the role of HPLC-DAD in the analysis of mephedrone and methcathinone, which was the selected synthetic cathinone for simultaneous analysis with mephedrone. Some articles were published after 2010 regarding qualitative and quantitative HPLC analysis of mephedrone and methcathinone.

In 2011, both Santali et al. and Maskell et al. developed and validated a quantitative HPLC-DAD method for mephedrone. Both studies validation parameters were satisfactory. However, the Maskell et al. method was more sensitive and takes less time. Santali et al. tested their own synthesized mephedrone salts, where the method was applied for the analysis of mephedrone alone or simultaneously with other adulterants commonly used in the illegal drug market, such as caffeine, paracetamol, lidocaine, ketamine, sucrose and lactose. In contrast Maskell et al. analysed mephedrone and other cathinones in post-mortem blood samples, where in many of these cases, mephedrone was attributed to be the main cause of death. Data collected by Maskell et al. showed concentrations of mephedrone in the range of (0.13 - 2.24 µg/ml) in blood samples. Santali used

nicotinamide as an internal standard, and Maskell used cinchonine. (Santali et al., 2011; Maskell et al., 2011).

In 2014, Golasik et al. qualitatively analysed urine samples taken from methcathinone long-term users by HPLC-DAD. The main aim of their work was to determine manganese in urine of long-term methcathinone users, however, methcathinone detection was carried via HPLC-DAD. Urine samples were extracted with ethyl acetate, evaporated and reconstituted with mobile phase before HPLC-DAD analysis (Golasik et al., 2014)

Adamowicz et.al. Analysed seized powders of mephedrone using HPLC-DAD and applied this method to analyse vitreous humour samples. Gradient elution of mobile phase consisting of ACN: water containing 100 µL 85% orthophosphoric acid per 1 L was used. Mephedrone-d3 was used as internal standard. Method was linear, with R2= 0.999. LOD and LOQ were 0.08 and 1 ng/ml, respectively (Adamowicz et al., 2013)

A summary of the most significant literature review data about the current role of HPLC in the analysis of mephedrone and related methcathinone is presented in Table 2.

2.2. Current role of chromatography-mass spectrometry in the analysis of selected NPSs and their metabolites in biological fluids

Chromatography techniques coupled to mass spectrometry have become an essential part of modern forensic laboratories as screening as well identification tools. Utilizing the highly specific and accurate mass spectrometry instruments has significantly increased the analytical efficiency of the forensic laboratories. However, the problem in the analysis of NPSs and their metabolites using Chromatography-Mass spectrometry is mainly due to their wide range that fluctuates from newly synthesized molecules to herbal psychoactive substances. Additionally, pure standards of these compounds and their metabolites are not always available, and there is not enough data available about their chromatographic features up until late after their appearance (Favretto et al., 2013).

Electron-impact MS (EI-MS) is the typical method used for the identification of illegal drugs, using the NIST Mass Spectral Library or other data bases. However, most designer drugs are not contained

within these libraries. GC-MS is the most commonly used analytical technique in forensic laboratories for analysis of designer drugs as most of these designer drugs have volatile properties. GC most of the time needs a relatively short period of time for analysis. The usual column is non-polar DB-5MS [(5%-phenyl)-methylpolysiloxane] or an analogue, as it has high separation efficiency.

2.2.1. Currently available analytical studies of the selected NPS using LC-MS

Systematic toxicological analysis of serum collected in an emergency department from patients with history of acute mephedrone toxicity was performed by Wood et.al. They combined GC-MS and LC-MS/MS to confirm qualitatively the presence of mephedrone. For LC-MS, MS in Multiple Reaction Monitoring (MRM) analysis were utilized to confirm the fragments of mephedrone of m/z: 178, 160 145 and 119 (Wood et al., 2010).

Sorenson studied the simultaneous quantitative analysis of mephedrone and some other cathinone derivatives in human whole blood samples of both live and post-mortem cases, developed a liquid chromatography–tandem mass spectrometry method via pneumatically aided electrospray ionization. Methanol was used, as a pre-treatment step for protein precipitation, supernatant was ultra-filtered and mixed with deionised water before injection onto the LC-MS system. Method was validated, with LOD of 0.5-3 ng/ml, recovery percent of 87-106% and SD values better than 7% at 20 ng/ml. For the stability of cathinones, extracts were acidified with formic acid (Sorensen, 2011).

Jankovics et al. developed a LC–MS/MS method for screening of mephedrone, methcathinone and other five-methcathinone analogues. Samples source were unknown bulk powders that were claimed to be harmless substances, which turned to contain at least methcathinone or one of the studied methcathinone derivatives. Separation was carried on reverse phase chromatography with gradient mode elution. DAD was used to record UV spectra (210-240 nm), and MS in MRM mode was utilized to identify fragmentation characteristics. The fragmentation characteristics were discussed thoroughly in this work in comparison with previously published work. Limit of detection was 2 ng/mL, and total run time was less than 8 min. Typical fragmentation characteristics of the studied compounds were discussed (Jankovics et al., 2011).

Shah et al. developed and validated a quantitative analysis study of mephedrone and its metabolites 4-methylephedrine and 4-methylnorephedrine by LC-MS/MS, and applied it to human hair samples. Method was proven to be linear in the range 5–100 pg/mg for mephedrone and 10–150 pg/mg for the two metabolites. They used LLE for sample preparation using hexane for mephedrone and mixture of chloroform, ethanol and diethyl ether (3:1:1 ratio) for the metabolites. Precision, accuracy, limit of detection, limit of quantitation and extraction recovery were determined and all were within limits specified by FDA guidelines. LOD was 2.5 pg/mg and LOQ was 5 pg/mg. For the metabolites LOD and LOQ were 5 pg/mg and 10 pg/mg, respectively (Shah et al., 2012).

Pedersen et al., in 2012, performed analysis of blood and urine samples of forensic cases using UPLC-MS/MS and UPLC-QToF/ MS, they could detect mephedrone and its metabolites in blood, the mephedrone concentration in blood samples was 1-51 µg/kg, and up to 9 µg/kg for its metabolite hydroxyl tolyl mephedrone, in one of the cases the concentration of mephedrone was as high as 700 µg/kg, and 190 µg/kg for hydroxyl tolyl mephedrone (Pedersen et al., 2013).

Swortwood et al. analysed a number of cathinones, including mephedrone, and other designer drugs in serum samples by a quantitative LC -MS/MS analytical method. Samples were prepared by SPE, LOD for all analytes was found to be about 10 pg/mL, while LOQ values ranged from 1 to 10 ng/mL (Swortwood et al., 2013).

In 2013, Marinetti and Antonides developed an LC-MS method for analysis of synthetic cathinones, including mephedrone, in blood and urine samples collected from real cases. Samples were prepared by LLE (Marinetti and Antonides, 2013)

For the study and analysis of synthetic cathinone and two piprazines, including mephedrone and 4-fluoromethamphetamine, de Castro et al. applied and validated an LC-MS/MS for analysis in oral fluid. Samples were SPE extracted and chromatographic separation was achieved using 0.1% formic acid in acetonitrile mobile phase. The method showed to be linear over the specified range of 0.2/0.5-200 ng/ml with LOD and LOQ of 0.025-0.1 ng/ml and 0.2-0.5 ng/ml respectively with recovery percent of 87.9-134.3 (de Castro et al., 2014).

Elian et al. analysed a number of amphetamines and cathinones, including mephedrone, in human hair samples by LC-MS/MS. Sample preparation was performed by SPE, mobile phase contained acetonitrile and formic acid, gradient chromatography mode was performed using C_{18} column. Run time was less than 5 minutes (Elian et al., 2014).

In 2014, Reid et al. analysed amphetamine-like drugs including mephedrone, synthetic cannabinoids and their urine metabolites in sewage using a quantitative UHPLC-MS/MS, where samples collected from sewage of three Norwegian cities. LOD was 1 ng/L for mephedrone and other amphetamine-like drugs, while LOQ was 3 ng/L (Reid et al., 2014)

In 2013, Paoli et al. purchased methoxetamine and other two psychoactive drugs through the internet under the label "research chemicals," and analysed them using GC-MS. They also developed and validated a quantitative HPLC-MS/MS method and HPLC-UV method for determination of these materials in urine, blood, and vitreous humour. The developed HPLC–UV method was found to be satisfactorily valid in the range between 0.16 and 5.0 mg/L (De Paoli et al., 2013).

Amaratunga performed quantitative analysis of cathinones in oral fluid. They applied UHPLC-MS/MS method, with gradient mode, and SPE for sample preparation. The method was validated and found to be accurate, precise, linear and sensitive, with acceptable recovery. The method was successfully applied on human oral fluid samples (Amaratunga et al., 2013).

In 2013, Meyer et al. applied GC-MS and LC-MS methods to analyse methoxetamine and its phases I and II metabolites in rat and human urine samples. Moreover, human CYP 450 enzymes were found to be responsible for the initial steps of methoxetamine metabolism *in vitro*. The last step was the analysis of human urine samples collected from patients. They could detect eight metabolites suggesting O-demethylation, N-deethylation or hydroxylation phase I metabolic pathways. Phase II metabolism, namely sulfation or glucuronidation, or combinations of more than one pathway were also suggested (Meyer et al., 2013).

In 2013, Saffar et al. developed and validated a LC–MS/MS method for screening of novel psychoactive drugs, including mephedrone and methoxetamine, in urine. Samples were diluted,

pethidine-d5 was used as an internal standard. Gradient elution mode was applied and run time was 4.0 min. Method was validated and found to be linear in the range of 0.1–10 µg/mL, with suitable precision where CV< 15% (Al-Saffar et al., 2013).

Elliot et al., in 2013, screened and confirmed presence of mephedrone and other cathinones in bath salts using direct sample analysis ToF/MS. They suggested the presence of this technique in forensic laboratories and replacing the commonly used lengthy GC/MS and LC/MS/MS methods techniques can be helpful as it does not need sample preparation and takes much less time for training and analysis and improves laboratory efficiency (Elliott et al., 2013).

Recently, Chen Applied a LC–MS method to quantitatively analyse methcathinone and other 3 designer drugs and some of their metabolites through *in vitro* study. He performed a metabolism study for each drug separately using microsomes S9 fraction prepared from rat liver. The selected drugs and five of their major metabolites were successfully detected. The method was validated, it was linear in the range from 0.01 to 5.0 g/mL, limits of detection were less than 0.03 g/mL where RSD values were below 5.9%, and recoveries were more than 77.4% (Chen, 2015)

Very recently in 2015, Pasin et.al developed and validated a quantitative LC-MS analytical method of novel designer drugs, including mephedrone and methcathinone, in post mortem blood samples. LLE with acetonitrile was used for sample preparation, mephedrone-d3 was the used internal standard, and run time was 15 minutes. Method was found to be linear in the range of 0.05–2 mg/L. LOD for analytes ranged from 0.007 to 0.07 mg/L, while LOQ values were in the range of 0.05–0.1 mg/L. Extraction recoveries were from 71 to 100% (Pasin et al., 2015).

Summary of the most significant literature review data about the currently available analytical studies of selected and related NPS using LC-MS are presented in Table 3.

2.2.2. Currently available analytical studies of NPS using GC-MS

Torrance and Cooper performed quantitative analysis of mephedrone. They used a certified standard of mephedrone from and the method was an accredited method for amphetamines taken from in

blood using GC–MS-EI and using derivatization with PFP. Selected ions monitored were m/z 204, 160 and 91 using the internal standard MDA-d5., and LOQ was determined to be 0.1 mg/L (Torrance and Cooper, 2010).

In 2010, Meyer et al. applied a GC-MS method to rat and human urine samples and detected metabolites of mephedrone and other Beta-keto amphetamines. They suggested the metabolic mechanisms of reduction of the ketone group to the corresponding alcohol, N-demethylation to give a primary amine, and the oxidation of the tolyl functionality to alcohol or carboxylic acid (Meyer et al., 2010).

Brandt et al. performed a relatively similar work, where they purchased a number of legal high products from different internet websites and performed qualitative analysis using GC-MS/ion trap and NMR spectroscopy. They dissolved samples in methanol, and applied methods that were used before for psychoactive drugs with related structure. The results were compared with reference standards, and they concluded that more than two thirds of the NRG-1 and NRG-2 products that were marketed legal as highs or as a replacement for banned mephedrone were truly containing banned cathinones (Brandt et al., 2010).

In 2011, Baron et al. performed analysis of seven legal high samples purchased from online traders, to validate the identity and purity of the labelled materials which claimed to contain either bath salts, research chemicals or plant food. They analysed samples using FTIR. Subsequently, GC-MS analysis was performed, after being extracted with methanol, run time was about 10 minutes. NIST spectra library and reference standards were utilized for comparison. The analytical study proves that only one sample contained what it was claimed on the label, while others contained different materials (Baron et al., 2011).

In 2011, Power et al. studied the chemical analysis of substituted cathinones using GC-MS, where the isomers 2-, 3- and 4-methylmethcathinone were analysed. They synthesized 2- and 3-methylmethcathinone. They found that the isomers can be separated by GC-MS without need to derivatization. They also analysed a seized sample by GC-MS, it contained mephedrone and

benzocaine with minimal amounts of 2- and 3-methylmethcathinone. Additionally, they performed NMR analysis, the results confirmed those obtained by GC-MS (Power et al., 2011).

In 2011, McDermott et al. studied mephedrone, ethcathinone and their isomers. The research team performed GC-MS analysis of drug samples assumed to contain mephedrone and ethcathinone, and they observed other compounds in the chromatogram. The initial proposal that these other compounds were isomers of mephedrone and ethcathinone was confirmed by the comparison and analysis of in-house synthesized isomers of these two designer drugs. They proposed these isomers are contaminants due to impure starting materials or rearrangement of the propiophenone originating products to the phenylacetone ones. In addition, they suggested that the presence of isomeric compounds might lead to common origin of the samples (McDermott et al., 2011).

In 2012, Martin et al. applied GC-MS using hair samples to prove mephedrone chronic toxicity. They extracted samples using ethyl acetate (after alkalinisation with 1 N NaOH). Applied derivatization using a mixture of heptafluorobutyric anhydride/ethyl acetate (100:50, v/v), Detection was achieved in single ion monitoring mode. The detected mephedrone concentrations ranging from 0.2 to 313.2 ng/mg. Method was linear, precise and accurate with LOQ of 0.2 ng/mg. The LOD was 0.08 ng/mg. The intra-day variations (CV) at two concentrations was less than 4%, while inter-day variation was less than 15% (Martin et al., 2012).

As a replacement for immunoassays screening methods, Lua et al. studied GC-MS analysis of mephedrone, ketamine and other amphetamine-type stimulants. They suggested that due to the limitations of availability of new immunoassays to keep with the rapidly emerging NPS market, GC-MS analyses are potentially one of the alternatives for the analysis of NPS for which no immunoassay kits are available (Lua et al., 2012).

Elie, et.al developed a rapid screening method for the analysis of 23 analytes, including methoxetamine. They analysed 35 products bought through internet, head shops, and successfully identified the active ingredients. The separation was achieved within 4 minutes using low bleed and

highly inert chromatography column, and their method showed to be efficient even with the unavailability of reference standards (Elie et al., 2013).

For the evaluation of whether designer drugs, especially designer amphetamines and cathinones, would interfere with currently existing and used amine confirmation methods, Holler et al. studied the effect of a number of synthetic amphetamines, including 4-fluoromethamphetamine, through immunoassays screening and utilizing MS and using three different derivatization methods. The initial proposal was that due to structural similarity, these designer amphetamines would potentially interfere with existing immunoassays and GC-MS confirmatory methods. They concluded the need of validation and revalidation of existing detection methods for amines to keep up with challenging rapidly appearing NPS (Holler et al., 2014).

Leffler et al., in 2014, analysed a number of cathinone analogues including mephedrone, in street samples, using GC–MS, HPLC and MS/MS. Methanol was used as extraction solvent, and GC–MS was applied for initial analysis, while direct infusion MS/MS was performed for identification of unknown molecules, and finally HPLC fractionation was used for accurate masses and information in order to predict structural formula of molecules. Standards were then purchased from Cayman chemical company and used as a proof of identity of the analytes using GC–MS. The claimed active materials of the designer drugs samples were found to constitute only 11% to 85% of the labelled amount (Leffler et al., 2014).

In 2014, Daeid et al. synthesized mephedrone and another 15 legal high cathinone derivatives and then developed a qualitative GC–MS method to analyse them. Samples were prepared by dissolving analytes in methanol, and direct injecting them into GC–MS system, without the need for derivatization. Cathinone mixtures with commonly added adulterants, which are lidocaine, benzocaine, and procaine, were successfully identified and separated (Daeid et al., 2014).

Very recently, Meng et al. performed analysis of selected drugs of abuse including methcathinone in blood and urine via GC-MS. The aim of the study was a comparison of different types of microextraction methods. Method was linear for the selected analytes in 0.0030–10 g/ml

concentration range. LOD was calculated in both methods to be 0.5 and 5 ng/ml and 0.5 to 4 ng/ml, respectively (Meng et al., 2015).

Summary of the most significant literature review data about the currently available analytical studies of selected and related NPS using GC-MS are presented in Table 4.

2.3. Currently available data for *in vitro* studies of selected NPS

Limited data are available regarding *in vitro* studies of the selected NPS and related drugs, and most of the available data was about identification of the metabolites of NPS followed by analytical techniques, mostly MS. A few other studies were concerned about the effects of NPS at the cellular level and to mimic potentially harmful effects *in vivo*.

Meyer et al performed *in vivo* metabolic studies of mephedrone, and applied GC-MS which enabled them to detect mephedrone metabolites in human and rat urine. They concluded that mephedrone may be metabolized through phase 1 enzymatic systems through demethylation, reduction or oxidization. Demethylation involves the removal of the N-methyl functional group producing nor-mephedrone which will go further into reduction of the ketone moiety producing the corresponding alcohol, nor-dihydro mephedrone Also, hydroxytolyl-mephedrone, 4-carboxy-dihydro mephedrone and nor-hydroxytolyl mephedrone were detected at their study as products of an overlapping metabolic pathway of oxidation, reduction and N-demethylation. It was concluded through their study also that phase II metabolic pathways are involved in the metabolism of mephedrone through sulphontaion or glucuronidation of the hydroxytolyl- mephedrone and its demethylated form (Meyer et al., 2010).

Pedersen et al. , in 2013, performed *in vitro* studies using CYP enzymes and human liver microsomes, followed by analysis of blood and urine samples of forensic cases using UPLC-MS/MS and UPLC-QToF/ MS , they found that mephedrone is mainly metabolized by CYP450 2D6 enzyme in phase I metabolism giving two major metabolites which are nor-mephedrone and hydroxyltolyl mephedrone, these two metabolites were identified in addition to other three metabolites, namely carboxy mephedrone, dihydro mephedrone and 4-carboxy-dihydro-mephedrone, the mephedrone

concentration in blood samples was 1-51 µg/kg, and up to 9 µg/kg for its metabolite hydroxyl tolyl mephedrone, In one of the cases the concentration of mephedrone was as high as 700 µg/kg, and 190 µg/kg for hydroxyl tolyl mephedrone (Pedersen et al., 2013).

Meyer et al. recently utilised GC-MS for *in vivo* studies and detection of MXE metabolites in human and rat urine. They concluded that MXE may be metabolised through phase I and phase II enzymatic systems. Phase I enzymatic reactions involve N-demethylation, O-demethylation and hydroxylation or a combination of these metabolic steps. It was concluded through their study also that phase II metabolic pathways are involved in the metabolism of MXE through sulphontaion or glucuronidation of most of these metabolites (Meyer et al., 2013).

Menzies et al. performed *in vitro* studies utilizing LC-MS for the analysis of MXE metabolites after incubation with human liver microsomes. Phase I and phase II metabolites were detected in their study, where phase I metabolites were the product of N-demethylation, O-demethylation, reduction, hydroxylation and dehydrogenation.

Regarding the *in vitro* metabolic studies of methcathinone, although it appeared in the market for drugs of abuse many years ago, only recently has there been published data about its metabolism. Recently, Chen published data about *in vitro* studies of metabolism of designer drugs including methcathinone. Cathinone was reported as a metabolite for methcathinone through N-demethylation (Chen, 2015).

A search for literature about *in vitro* studies on 4-fluromethamphetamine yielded no hits, and no published data could be found about the metabolic and toxic profile of 4-fluoromethamphetamine.

Table 2: Summary of significant analytical methods for analysis of mephedrone using HPLC-DAD

Reference	Analytes	Biological matrix	Sample preparation	Chromatography conditions	validation parameters
Santali et al., 2011	Mephedrone and adulterants	NA[1]	in methanol	Mobile phase: Gradient mode: phase A: methanol:10 mM ammonium formate (pH 3.5) (40:60); phase B: methanol Stationary Phase: ACE $3C_{18}$ Total Run Time: <10 minutes	Range of Linearity: 0.5–10 µg /mL Linearity: R^2> 0.999 Precision: 0.36 –0.90% LOD: 0.1 µg/ mL LOQ: 0.3 µg/ mL
Maskell et al., 2011	Mephedrone and other cathinones	Post-mortem blood	LLE with 1-hlorobutane	Mobile phase:10% acetonitrile (with 25mM TEAP buffer) Stationary Phase: C_{18} Total Run Time: <6 minutes	Range of Linearity: 0.078-10 µg/ml Linearity: R^2 > 0.999 Precision: within 3%. LOD: 0.039 µg/mL LOQ: 0.078 µg/mL
Adamowicz, 2013	mephedrone	NA	In methanol	Mobile phase: Gradiant.Acetonitrile: water containing 100 µL 85% orthophosphoric acid per 1 L Stationary Phase: LiChroCART 125-4 LiChrospher RP-select B Total Run Time: 14 min	Range of Linearity: 1-100ng/ml Linearity: R^2 0.996 Precision: 10.4-14.2 LOD: 0.08 ng/ml LOQ: 1 ng/ml
Golasik et al., 2014	Methcathinone	Urine of chronic abusers	LLE with ethyl acetate	Mobile phase: Gradient mode: phase A: 500 mL of deionized water with 100 µL of concentrated phosphoric acid; phase B: acetonitrile Stationary Phase: LiChroCART 125-4 LiChrospher RP-select B Total Run Time: 30 minutes	Range of Linearity: NDA[2] Linearity: NDA Precision: Not tested LOD: NDA LOQ: NDA

1: NA: Not applicable 2: NDA: No data available

Table 3: Summary of significant analytical methods for analysis of selected NPS using LC-MS

Reference	Analytes	Matrix	Sample preparation	Stationary phase	Chromatography	Mass spectrometry	validation parameters
Sorensen et al., 2011	Mephedrone, methcathinone and other cathinones	NA	protein precipitation, ultrafiltration Internal standard: Mephedrone-D3 methcathinone-D3	Agilent Zorbax EclipseXDBC-18 (75 mm x 4.6 mm)	Mobile phase: Gradient: Phase A: water Phase B: MeOH (both acidified with 0.1% formic acid) Stationary phase: Prodigy Phenyl-3 Total run time: <20 minutes	pneumatically aided electrospray ionisation, SRM	Range of linearity; 10–250 µg/L Linearity: R^2>0.997 Precision: SD <7% At 20 g/L LOD: 0.5-3 µg/mL LOQ: NDA
Jankovics et al, 2011	Mephedrone, methcathinone & its analogues	NA	Dissolved in methanol Internal standard: NDA[1]	Agilent, DB-1 (30 m×0.25 mm×0.25 µm)	Mobile phase: Gradient: Eluent A: 95% water, 5% ACN, 0.1% formic acid. Eluent B: 95% ACN, 5% water, 0.1% formic acid. Stationary phase: Agilent Zorbax EclipseXDBC-18 (75 mm x 4.6 mm) Total run time: <8 minutes	MRM mode	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: Average: about 2 ng/mL for all analytes LOQ: NDA
Pedersen et al., 2011	Mephedrone and its metabolites	Blood and urine	SPE Internal standard: Amphetamine-D5 and orphenadrine	Agilent SB-C_{18} column	Mobile phase: 0.1% formic acid (solvent A) and 100%acetonitrile (B), gradient Stationary phase: UPLC BEH C_{18} Total run time:	QToF, MRM, ESI	Range of linearity; 1 - 400 mg/kg for mephedrone Linearity: NDA Precision: CV < 10% LOD: NDA LOQ: NDA
Shah et al., 2012	Mephedrone and metabolites	Human hair	LLE with hexane Internal standard: Mephedrone-d3	Phenomenex, C_{18} (50 x 2 mm x4 µm)	Mobile phase: Acetonitrile: water, different conc., gradient mode Stationary phase: Agilent SB-C_{18} column Total run time: ~ 10 minutes	selective reaction monitoring (SRM) mode	Range of linearity; 5–100 pg/mg for mephedrone Linearity: R^2 >0.999 Precision: RSD %: 0.4-0.8 for mephedrone LOD: 2.5pg/ml for mephedrone LOQ: 5 pg/ml for mephedrone
Paoli et al., 2013	methoxetamine and other two psychoactive drugs	Blood, urine and vitreous humour	LLE with 1-chlorobutane Internal standard: NDA	C_{18}	Mobile phase: Gradient: soln. A: ammonium formate, formic acid Soln. B: acetonitrile, ammoniumFormate, formic acid Stationary phase: Phenomenex, C_{18} (50 x 2 mm x4 µm) Total run time: 20 minutes	MRM	Range of linearity; 0.16-5.0 mg/L Linearity: NDA Precision: RSD%= 1.62% LOD: 0.03 mg/L in blood, 0.04 mg/L in urine LOQ: 0.16 mg/L in all matrixes
Amaratunga et al., 2013	Mephedrone, methcathinone and other cathinones	Human oral fluid	SPE Internal standard: NDA	TF TG-1MS capillary column	Mobile phase: Mobile phase A: 0.1% formic acid in water: acetonitrile (95: 5, v/v), mobile phase B: 0.1% formic acid in acetonitrile Stationary phase: C_{18} Total run time:	ESI+, multiple reaction monitoring(MRM) mode	Range of linearity; 1.0 –500.0 ng/mL Linearity: R^2 >0.990 Precision: NDA LOD: NDA LOQ: 1.0 ng/mL
Meyer et al., 2013	methoxetamine and its phases I and II metabolites	rat and human urine	SPE Internal standard: ketamine	1MS capillary column	Mobile phase: helium Stationary phase: TF TG-1MS capillary column Total run time: About 20 minutes	Electronic ionization (EI) and positive-ion chemical ionization (PICI)	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: NDA, LOQ: NDA

Continued

Reference	Analytes	Matrix	Sample preparation	Stationary phase	Chromatography	Mass spectrometry	validation parameters
Saffar et al., 2013	Mephedrone, methoxetamine and other NPS	urine	dilution Internal standard: pethidine-d5	C_{18} column	Mobile phase: CHECK Stationary phase: Ethylene Bridged Hybrid (BEH) C_{18} Total run time: 4 minutes	LC-MS/MS	Range of linearity; 0.1–10 µg/mL Linearity: NDA Precision: LOD and LOQ: NDA
De Castro et al., 2014	mephedrone and 4-fluoromethamphetamine	Oral fluid	SPE Internal standard: NDA	C_{18}	Mobile phase: formic acid 0.1% and acetonitrile Stationary phase: T3 column Total run time: NDA	CHECK	Range of linearity; 0.2/0.5-200 ng/ml Linearity: R^2 >0.990 Precision: %CV = 0.0–12.7% LOD: 0.025-0.1 ng/ml LOQ: 0.2-0.5 ng/ml
Elian et al., 2014	Mephedrone, other amphetamines and cathinones	human hair	SPE Internal standard: mephedrone-d3	Acquity UPLC BEH C_8 column	Mobile phase: acetonitrile and formic acid Stationary phase: C_{18} Total run time: 5 minutes	Tandem mass spectroscopy	Range of linearity; 0.1-10 ng/mg Linearity: R^2 > 0.995 Precision: As percent recovery: 106 ± 7, for 2ng/mg LOD: 0.05 ng/mg LOQ: 10 ng/mg
Reid et al., 2014	Mephedrone and other amphetamine-like drugs and their urine metabolites	Sewage samples	SPE Internal standard: mephedrone-d3	Waters, C_{18} column (2.1x150 mm,1.8 mm)	Mobile phase: Gradient: Solvent A: NH_4AC 10 mM and 0.1 % acetic acid in water. Solvent B: NH_4AC10 mM & 0.1 % acetic acid in MeOH Stationary phase: Acquity UPLC BEH C_8 column Total run time: NDA	MRM	Range of linearity; 1.6 to 50 ng/L Linearity: R^2 > 0.99 Precision: RSD% of recover 5-40 % LOD: 1 ng/L LOQ: 3 ng/L
Pasin et.al, 2015	mephedrone and methcathinone and other drugs	Spiked blood samples	LLE with acetonitrile Internal standard: mephedrone-d3	C_{18}	Mobile phase: Gradient: A: (5 mM Ammonium Formate), B [Acetonitrile, 0.1% (v/v) Formic Acid]: Stationary phase: Waters, C_{18} column (2.1x150 mm,1.8 mm) Total run time: 15 minutes	positive electrospray ionization mode	Range of linearity; 0.05–2 mg/L Linearity: R^2> 0.98 Precision: % CV <15 LOD: 0.007 to 0.07 mg/L LOQ: 0.05–0.1 mg/L
Chen, 2015	Methcathinone, other designer drugs and them *in vitro* metabolites	*in vitro* drug metabolites	Extraction by acetonitrile Internal standard: NDA	C_{18}	Mobile phase: phase A (water, 10 mM ammonium acetate, 0.1% acetic acid) and B (methanol) Stationary phase: C_{18} Total run time: ~10 minutes	Ion trap	Range of linearity; 0.08- 5.0 (µg/mL) Linearity: R^2=0.9991 Precision: For inter and intra-days< 5.9% LOD: 0.03 µg/mL LOQ: 0.08 µg/mL

1: NDA: No data available

Table 4: Summary of significant analytical methods for analysis of selected NPS using GC-MS

Reference	Analytes	Analytical method	Matrix	Sample preparation	Chromatography	Mass spectrometry	validation parameters
Wood et.al., 2010	Mephedrone	GC-MS and LC-MS/MS quantitative	Serum from patients	Internal standard: MDMA D5	Mobile phase: Methanol, deionized water, Stationary phase: HP5MS column Total run time: 12 minutes	MRM mode	Range of linearity; 0.01 to 1 mg/L Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA
Meyer et al., 2010	Mephedrone, other Beta-keto amphetamines and their metabolites	GC-MS	rat and human urine	NDA Internal standard: NDA	Mobile phase: NDA Stationary phase: NDA Total run time:	NDA[2]	Range of linearity; Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA
Torrance and Cooper, 2010	mephedrone	GC–MS-EI, quantitative	NA[1]	Post mortem blood, urine and hair Internal standard: MDA-d5	Mobile phase: According to ISO/IEC 17025 accredited method Stationary phase: According to ISO/IEC 17025 accredited method Total run time: Not mentioned	ToF	Range of linearity; Not tested Linearity: Not tested Precision: Not tested LOD: 0.1 mg/L LOQ: 0.1 mg/
Meyer et al., 2010	Mephedrone, other Beta-keto amphetamines and their metabolites	GC-MS	rat and human urine	SPE Internal standard: NDA	Mobile phase: Helium Stationary phase: HP-1 capillary (12 m×0.2 mm ID) Total run time: About 16 minutes	EI mode	Range of linearity; Not tested Linearity: Not tested Precision: Not tested LOD: Not tested LOQ: Not tested
Baron et al., 2011	Unknown legal highs	GC-MS	NA	Extraction with methanol Internal standard: NDA	Mobile phase: Helium Stationary phase: Agilent, DB-1 MS column (30 m×0.25 mm×0.25 µm) Total run time: 10.25 minutes	total ion count (TIC) mode	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA
Power et.al, 2011	Mephedrone and other ring substituted isomers	GC-MS	NA	Dissolved in methanol Internal standard: NDA	Mobile phase: helium Stationary phase: HP-ULTRA 1 capillary column Total run time: 20 minutes	NDA	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA
McDermott et al., 2011	Mephedrone, methcathinone and their isomers	GC-MS	NA	Dissolved in methanol Internal standard: NDA	Mobile phase: helium Stationary phase: HP-ULTRA 1 capillary column Total run time: 20 minutes	NDA	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA

Continued

Reference	Analytes	Analytical method	Matrix	Sample preparation	Chromatography	Mass spectrometry	validation parameters
Martin et al, 2012	mephedrone	GC-MS	hair	Extraction with ethyl acetate Internal standard: NDA	Mobile phase: NDA Stationary phase: NDA Total run time: NDA	NDA	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: 0.08 ng/ml LOQ: 0.2 ng/ml
Martin et al, 2012	mephedrone	GC-MS	hair	Extraction with ethyl acetate Internal standard: NDA	Mobile phase: NDA Stationary phase: NDA Total run time: NDA	NDA	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: 0.08 ng/ml LOQ: 0.2 ng/ml
Lua et al, 2012	Mephedrone, ketamine and other amphetamine-like stimulants	GC-MS	NA	NDA Internal standard: NDA	Mobile phase: NDA Stationary phase: NDA Total run time: NDA	NDA	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA
Holler et al., 2014	4-fluoromethamhetamine and other amphetamines	GC-MS, qualitative	urine	SPE Internal standard: NDA	Mobile phase: helium Stationary phase: DB-5MS Total run time: NDA	NDA	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA
Daeid et al., 2014	Mephedrone and other legal highs and adulterants	GC-MS, qualitative	NA	Dissolved in methanol Internal standard: eicosane	Mobile phase: helium Stationary phase: capillary column HP5 MS Total run time: 10 minutes	mass selective detector (MSD)	Range of linearity; NDA Linearity: NDA Precision: NDA LOD: NDA LOQ: NDA
Meng et al., 2015	methcathinone	GC-MS	blood and urine	microextraction methods Internal standard: NDA	Mobile phase: NDA Stationary phase: NDA Total run time: NDA	NDA	Range of linearity; 0.0030–10 g/ml Linearity: NDA Precision: NDA LOD: 0.5 to 5 ng/ml LOQ: 0.5 to 4 ng/m

1: NA: Not applicable 2: NDA: No data available

Chapter 3 Materials and Methods

3.1. Analysis of mephedrone and other selected drugs of abuse utilizing HPLC after extraction from biological samples

The HPLC technique is based on a solid (stationary) phase and a liquid (mobile) phase, which contrasts with Gas Chromatography (GC), where the technique is based on a gaseous (mobile) phase. HPLC is superior for the analysis of non-volatile or unstable organic compounds (Engelhardt, 2004; USP-NF, 2007b). HPLC is the core instrumentation used for analysing non-volatile drugs of abuse (Mc Fadden et al., 2006), where reversed phase chromatography (RPC) is the main HPLC mode used. It is a combination of a non-polar stationary phase (the column), and a polar mixture of water and an organic solvent-mobile phase (Snyder et al., 2009). HPLC is one of the most common available analytical instruments in forensic and analytical laboratories. Reversed phase chromatography is the most common form of liquid chromatography and it is advantageous for its compatibility with aqueous samples and flexibility in modifying the mobile phase, with either organic modifiers or buffer solutions (Snyder et al., 2009; McCalley, 2010).

In method development utilizing chromatography, the selection of suitable stationary phases is of prime importance, since it contributes differential interactions with components of a mixture resulting in their separation (Snyder et al., 2009). The type of stationary phase suitable for the analysis is dependent on the physicochemical characteristics of the analytes of interest. The column length affects separation efficiency, working pressure and analysis time. Other column variables - e.g., column diameter, packing material - are equally important and affect analytical method parameters differently - separation efficiency, working pressures, sample amount … etc. (Bayne and Carlin, 2010; McCalley, 2010).

The composition of the mobile phase is also a core element that affects the separation. For reversed phase, the most common mode used in HPLC analytical methods, the mobile phase is usually a mixture of aqueous solution with one or more organic modifier. When choosing the organic modifier, parameters such as purity, detector compatibility, solubility of the sample, low viscosity and chemical inertness should be taken into consideration, in addition to the possible hazards using some types of solvents. The separation in HPLC is a combination of differential interactions of the analytes

with both stationary phase and mobile phase. Buffering the mobile phase is very important, as this assures reproducible retention peaks.

The use of a suitable internal standard for chromatographic assays is highly recommended. The internal standard must have physicochemical properties as similar to the drugs of interest as possible, and it must be added to the sample at the earliest possible stage in the method, before pH modification and extraction of the sample (SOFT/AAFS, 2006). The diode array detector (DAD) is one of the most commonly available detector types in forensic laboratories, and it is able to provide more spectral information than the fixed UV/Vis detector.

3.1.1. Materials

Mephedrone was purchased legally from web-based companies before it was declared illegal (plantfeedchemicals.com). The percentage purity of mephedrone was confirmed by Fourier transform infrared spectroscopy (FTIR). Mass spectrometry (MS) was used to verify the structure. Methcathinone was synthesized and identified in the analytical laboratory according to a previously published method (DeRuiter et al., 1994). Caffeine, monobasic potassium phosphate was from Sigma (UK), potassium chloride, (Fisher, UK). Methanol of analytical grade, acetonitrile (HPLC grade), boric acid (100.04%), n-butanol (99.96%), dichloromethane (99.88%), sodium acetate trihydrate (99-100.5%), potassium chloride (100.5 %), glacial acetic acid (99.88%), sodium hydroxide (98.9%) were all from Fisher Scientific. 2-acetamidophenol (≥97.0%) and nicotinamide (98.5%) were from Fluka (UK). Whole blood sample and blank horse blood was form oxoid limited (UK). Blank foetal bovine serum from Gibco® (UK).

3.1.2. Equipment

Electronic balance from Sartorius (Germany), Ultrasonic bath was from Kerry (UK) and centrifuge model was Allegra X-22 from Beckman Coulter™ (UK). Sample concentrator model was DRI-BLOCK®, DB.3A from TECHNE (UK), vacuum manifold from Agilent (USA), shaking water bath was from Grant Instruments (UK), Mikro 20 Hettich Zentrefugen Microcentrifuge (USA), Beckman Coulter Ultracentrifuge (USA).

HPLC components were from Merck Hitachi LaChrom (USA) and consisted of an interface D-7000, pump L-7100, an auto sampler L-7200 and a Diode Array Detector L-7455. Data acquisition and analysis were performed using EzChrome software. Phenomenex® (C_{18}, 150 ×4.6 mm, 5µm) separation column was from Phenomenex (USA)

3.1.3. Methods

3.1.3.1. Developing HPLC method (I) for the analysis of mephedrone

For primary qualitative and quantitative analysis of mephedrone utilizing HPLC-DAD, a master stock solution was prepared in deionised water at 10 times the maximum concentration used (10x20 µg/ml). Maximum standard concentration was prepared by topping up 100 µL with 900 µL deionized water to achieve the final concentrations of mephedrone 20 µg/ml and other standards concentration (range 2-20 µg/ml) were prepared by dilution of maximum standard concentration with water to achieve the desired concentration. Standards were then injected onto HPLC to determine the retention time and validate the linearity of the method. Mobile phase consisted of H_2O: ACN: Acetic Acid (85:10:2.5) under isocratic elution conditions of 1 ml/minute. The column used for analysis was Phenomenex® (C_{18}, 150 ×4.6 mm, 5µm); temperature was ambient; DAD recording spectral data wavelength were between 200 and 400 nm; injection volume was 20 µL and total run time was 30 minutes.

To experiment on the effect of different solution on the retention time, peak shape and maximum absorbance of the mephedrone, mobile phase was used to prepare standards. New standards maximum concentration was prepared by topping up 100 µL with 900 µL mobile phase solution to achieve the final concentrations of mephedrone 20 µg/ml and other concentration standards (range 2-20 µg/ml) were prepared by dilution of maximum concentration standard with mobile phase to achieve the desired concentrations. New standards were injected onto HPLC to experiment the effect of changing the solution. HPLC condition were the same but total run time was adjusted from 30 to 10 minutes as it was expected that mephedrone will elute in less than 10 minutes.

Two internal standards were used, which are acetamidophenol and nicotinamide, to optimize the analysis. The choice of these two internal standards was due to their structural similarity to

mephedrone and the expectation that they will behave similarly later when developing extraction methods. Acetamidophenol was prepared as stock solution of 80mg/L and 100 µL added to each sample. Nicotinamide was prepared as stock solution of 20 mg/L and 100 µL added to each sample HPLC condition were the same but different flow rates were experimented obtain the best resolution between the analytes. Accordingly, total run time was adjusted to keep with adjustments of flow rate (Table 5).

Table 5: Summary of the key trials performed to optimize the developed HPLC-DAD method (I) for the analysis of mephedrone

Chromatography conditions	FR[1]	I.S[2]
Mobile phase: H_2O: ACN: Acetic Acid (85:10:2.5). Column: Phenomenex® (C_{18}, 150 ×4.6 mm, 5µm). Isocratic elution, ambient temperature DAD spectral scan range: 200-400 nm	1	Acetamidophenol
	0.2	Acetamidophenol
	0.5	Acetamidophenol
	1	Nicotinamide

1: mobile phase flow rate (ml/min) 2: internal standard

Spectral scan was performed for the analytes to identify the maximum absorbance. For qualitative analysis, wavelength of maximum absorbance and retention time was used as qualifiers for identification. For quantitative analysis peaks the wavelength of maximum absorbance of mephedrone of 262 nm was used to integrate the peaks. A summary the optimised HPLC method parameters for the analysis of mephedrone are presented in Table 6.

Table 6: Optimized HPLC method (I) parameters for the analysis of mephedrone

Stationary phase	Chemically modified $C_{18,}$ 150 mm, 4.6 mm, 5 µm
Mobile phase	H_2O: ACN: Acetic Acid (85:10:2.5)
Temperature	Ambient
Elution	Isocratic
Flow rate	1 ml /min
Total run time	10 minutes
Internal standard	Nicotinamide (100µl of 20mg/L into 1 ml sample)

3.1.3.2. Experimentation of LLE of mephedrone from whole blood samples followed by analysis applying the optimized HPLC-DAD method (I)

Liquid-liquid extraction (LLE) of mephedrone was experimented followed by analysis of the extracts using the previously optimized HPLC method (I) – see Table 6 above. Different pH buffer solutions and different extraction solvents were used to evaluate the optimum extraction parameters.

Standard Buffer solutions were prepared following USP general recommendations (USP-NF, 2007a). Mixture solutions of boric acid and potassium chloride (H_3BO_3 /KCl) were prepared by dissolving 618.4 mg of H_3BO_3 (MW=61.83) and 745.5 mg of KCl (MW-74.55) in water and then diluting with water to 50 mL to produce a 0.2 M mixture solution. Sodium hydroxide (NaOH) solutions were prepared by dissolving 400 mg of NaOH (MW=39.997) in water and then diluting with water to 50 mL.to produce 0.2 M solution. Monobasic potassium phosphate (KH_2PO_4) solutions were prepared by dissolving 1.361 mg of KH_2PO_4 (MW=136.09) in water and then diluted with water to 50 ml to produce 0.2 M solution. To prepare pH=10 buffer solution, to 50 ml of H_3BO_3 /KCl (0.2 M) mixture solution added 43.7 ml of NaOH solution (0.2 M) and topped up to 200 ml with deionized water. To prepare pH=7 buffer solution, to 50 ml of KH_2PO_4 solution (0.2 M) added 29.1ml of NaOH solution (0.2 M) and topped up to 200 ml with deionized water.

Master stock solutions of the drug mephedrone were prepared in deionised water at 10 times (10x) the concentration needed, i.e. (10, 20, 30, 40 and 50 µg/ml). Whole blood samples were spiked with the drug mephedrone, by adding 100µL of 10x mephedrone stock solutions to 900 µL mL blood achieving final concentrations of 1, 2, 3, 4, or 5 µg/mL. 100 µL of the internal standard nicotinamide reference solution (20 µg/mL) was added. Whole blood samples were buffered by adding 1 ml of one type of buffer solution to determine the best pH value, and extraction was performed by adding 5 ml of one type of extraction solvents. Samples were then agitated via vortex mixer for 1 minute and finally centrifuged at 3500 rpm for 10 minutes. The organic layer was removed into a clean glass vial via a pipette and evaporated to dryness using a sample concentrator under streaming N_2 at 40 ^oC. The residue was reconstituted with 1 mL of the mobile phase, sonicated, filtered through 0.45 µm filters and then injected onto the HPLC system under the optimized condition (Table 7).

Table 7: Summary of the key experimentation performed for LLE of mephedrone from blood samples

Extraction solvent	Modifying buffer pH
Ethyl acetate	3
Ethyl acetate	7
Ethyl acetate	10
Hexane	10

3.1.3.3. Validation of the optimized method of LLE of mephedrone from blood samples followed by analysis utilizing HPLC-DAD

Master stock solutions were prepared at 10 times (10x) of the desired concentrations, i.e. (20,40,60,80 and 100 µg/ml). Whole blood samples were spiked with the drug mephedrone, by adding 100µL of 10x mephedrone stock solutions to 900 µL mL blood achieving final concentration in the range of (2-10 µg/ml), then added 100 µL of the internal standard nicotinamide reference solution (20 µg/mL).

The optimized LLE method for mephedrone was the following: Samples were buffered by adding 1 ml of Borate buffer pH 10 buffer and extraction was performed by adding 5 ml of ethyl acetate. Samples were then agitated via vortex mixer for 1 minute and finally centrifuged at 3500 rpm for 10 minutes. The organic layer was transferred into a clean glass vial via a pipette and then evaporated to dryness using a sample concentrator and streaming N_2 at 40 ºC. The residue was reconstituted with 1 mL of the mobile phase, sonicated, filtered through 0.45 µm filters and then injected onto the HPLC system.

Linearity of the HPLC method was assessed by preparing new standards in mobile phase in the range of (2-10µg/ml), injection onto the HPLC-DAD under optimized conditions and plotting the peak area ratios against corresponding concentrations.

Linearity of the analytical method was assessed by preparing pre-spiked whole blood samples with known concentrations of the drug mephedrone and the internal standard nicotinamide. Five standards were prepared within the working range (2-10 µg/mL), each standard was injected onto HPLC system in triplicate, and each peak area of the analyte mephedrone and the internal standard nicotinamide was integrated and the peak area ratio (PAR) calculated and plotted against corresponding

concentrations. Repeatability of the analytical method was assessed by preparing standards (n=5) of the drug mephedrone at 100% of the maximum range (i.e. 10 µg/mL), extracted the same day following the optimized LLE protocol and injected onto the HPLC-DAD under the specified conditions.

Intermediate precision was assessed by preparing standards of the drug mephedrone at 100% of the maximum range (i.e. 10 µg/mL) extracted over different days (n=3) following the optimized LLE protocol and injected onto the HPLC-DAD under the specified conditions.

Accuracy was assessed by calculating percent recovery by computing the ratio of peak area of each concentration against the peak area of the corresponding standard concentration.

Limit of detection and limit of quantification were calculated mathematically following these equations

$$LOD = \frac{3Cs}{\left(\frac{hs}{2hn}\right)} \qquad \textbf{Equation 8}$$

$$LOQ = \frac{10Cs}{\left(\frac{hs}{2hn}\right)} \qquad \textbf{Equation 9}$$

Where: *Cs*=concentration of the sample, *hs*: height of measured peak and *hn*: height of background noise (Bayne and Carlin, 2010).

3.1.3.4. Applying and validation of the optimized LLE followed by HPLC-DAD for simultaneous analysis of mephedrone, d-amphetamine, codeine, caffeine and ketamine from blood sample

The optimized LLE protocol from blood sample was used here with the following minor modification: whole blood samples were spiked with the specified drug, by adding 200 µL of 10x drug stock solutions to 1800 µL blood to achieve the desired final concentration (Table 8), then added 100 µL of the internal standard nicotinamide reference solution (20 µg/mL).

Table 8: Final concentrations for simultaneous analysis of the selected drugs.

	Mephedrone[1]	Codeine[1]	Caffeine[1]	d-amphetamine[1]	ketamine[1]
1	0.2	20	0.2	10	10
2	0.5	30	0.5	15	15
3	1	40	1	20	20
4	2	50	2	25	25
5	5	60	5	50	50

1: µg/mL

Samples were buffered by adding 1 ml of borate buffer pH=10 and extraction was performed by adding 5 ml of ethyl acetate. Samples were then agitated via vortex mixer for 1 minute and finally centrifuged at 3500 rpm for 10 minutes. The organic layer was transferred into a clean glass vial via a pipette and then evaporated to dryness using sample concentrator and N_2 at 40 ºC. The residue was reconstituted with 750 µL of the mobile phase, sonicated, filtered through 0.45 µm filters and then injected onto the HPLC system.

Precision of the analytical method was assessed through intermediate precision by computing RSD% for different samples prepared and extracted over different days.

Accuracy was assessed by calculating percent recovery by computing the ratio of peak area of each concentration against the peak area of the corresponding standard concentration.

3.1.3.5. Optimized HPLC method for the simultaneous analysis of mephedrone and methcathinone

The previously developed HPLC method was applied with some optimization. Standard Buffer solutions were prepared following USP general recommendation (USP-NF, 2007a). Acetic acid solution was prepared by adding 11.6 ml glacial acetic to water to make 100 ml of 2N acetic acid solution. To prepare pH=4.1 acetate buffer solution, 1.5 g of sodium acetate ($NaC2H_3O_2.3H_2O$) was placed in 1000-ml volumetric flask, added 19.5 of 2N acetic solution ad diluted with deionised water to make 1000 ml acetate buffer pH 4.1 solution. The new optimized HPLC method (II) conditions were: mobile phase consisted of acetate buffer of pH=4.1: ACN (85:15) under isocratic elution conditions at flow rate of 1 ml/min. The column used for analysis was Phenomenex® (C_{18}, 150 ×4.6 mm, 5µm); temperature was ambient; DAD recording spectral data wavelength were between 200

and 400 nm; injection volume was 20 µL and total run time was 10 minutes. Aniline was used as internal standard.

3.1.3.6. Optimized LLE method of mephedrone and methcathinone mixture followed by analysis utilizing HPLC method (II)

Standard Buffer solutions were prepared following USP general recommendation (USP-NF, 2007a). Mixture solutions of boric acid and potassium chloride (H_3BO_3 /KCl) were prepared by dissolving 618.4 mg of H_3BO_3 (MW=61.83) and 745.5 mg of KCl (MW-74.55) in water and then diluted with water to 50 mL to produce 0.2 M mixture solution. To prepare pH 9.2 buffer solution, to 50 ml of H_3BO_3 /KCl (0.2 M) mixture solution added 26.4 ml of NaOH solution (0.2 M) and topped up to 200 ml with deionized water. Master stock solution of drug and mephedrone were prepared at 10 times(10x) the concentrations used in water (i.e. 0.4, 1, 2, 4, 20, 50 and 100 µg/ml). To prepare working sample, 100 µL of the 10x concentration added to 900 µL of whole blood or serum.

In a separation funnel, 1 mL of pre-spiked blood (or serum) samples with known concentrations of mephedrone and methcathinone was taken; 50 µL of the internal standard aniline (0.25% in ACN: H_2O, 80:20, v/v) were added to the sample, and pH was modified by adding 1 mL of Borate buffer solution pH=9.2. The sample was extracted by 5 mL of the extraction solvent (Dichloromethane: n-Butanol, 8:2. v/v) sample solution for 15 minutes. The organic layer was transferred to a clean glass tube, re-extracted with 1 mL of 0.01M H_2SO_4, shaken, and then centrifuged for 10 minutes at 3500 rpm. The aqueous layer was filtered and injected onto HPLC system. All samples preparations were repeated over different days, and HPLC injections were carried out in triplicates.

3.1.3.7. Validation of the LLE method of mephedrone and methcathinone mixture followed by analysis utilizing HPLC method (II

Linearity: Linearity of the HPLC-DAD method (II) was assessed by injecting different concentration of standard solutions onto the HPLC system. Standard solution with concentrations covering the working range (0.1, 0.2, 0.4, 2, 4 and 10 µg/mL) of both analytes were prepared. Each prepared standard solution was filtered through 0.45 µm membrane filters before it was automatically injected.

A plot of peak area ratios against corresponding concentrations was produced, and least square regression analysis was performed

Precision: Precision of the analytical method included repeatability (intra-day variation) and an intermediate precision (inter-day variation). Repeatability was assessed by computing the RSD% values for different samples prepared and extracted in the same day(n=5), containing 5 µg/mL of each of the analytes. Intermediate precision was assessed by computing the RSD% for different samples prepared and extracted in different days, containing 5 µg/mL of each of the analytes.

Accuracy: Accuracy of the analytical method was assessed by calculating recovery percentages of the tested drugs at different days against drug controls. Drug controls were prepared at different concentration to allow the calculation of recovery percentages (ICH, 2005).

Specificity: Specificity of the HPLC method test was performed by assessment of sufficient resolution in addition to estimation of peak purity for the analytes. Peak purity was calculated by taking absorbance values for the peaks at 240 and 280 nm wavelengths and computing the ratio at every point across the peak. Constant ratios indicate pure peaks (i.e. no interference), and so specificity to the corresponding analytes (Papadoyannis and Gika, 2005; Snyder et al., 2009).

Limit of detection and Limit of quantification were calculated mathematically following previously mentioned equations. Equation 8 (p.72) was used to calculate LOD and Equation 9 (p.72) was used to assess LOQ for both analytes (Bayne and Carlin, 2010).

3.2. *In vitro* metabolism studies of selected NPS using pig liver microsomes followed by analysis utilizing LC-MS

3.2.1. Materials

Mephedrone and methoxetamine were purchased legally from web-based companies before any of them were declared illegal. Methcathinone was synthesized and identified in the analytical laboratory according to a previously published method (DeRuiter et al., 1994). MDAI was from LGC (UK). Ethylenediaminetetraacetic acid (EDTA), glycerol and sodium pyrophosphate was all from Sigma (UK), potassium chloride (100.5 %), was from Fisher Scientific. Tris Buffer saline from Biorad (UK)

Pig liver was obtained freshly from a local abattoir, kept ice cold and transferred directly to the laboratory for processing. For the NADPH regeneration system: nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase enzyme (G6PD) and glucose-6-phosphate (G6P) were purchased from Sigma (UK). Deionized water was used in all preparations, filtered and degassed prior to use when needed.

3.2.2. Equipment

An Electronic balance from Sartorius (Germany), Ultrasonic bath was from Kerry (UK) and centrifuge model was Allegra X-22 from Beckman Coulter™ (UK). Sample concentrator model was DRI-BLOCK®, DB.3A from TECHNE (UK), shaking water bath was from Grant Instruments (UK), Teflon mortar & pestle (UK), Mikro 20 Hettich Zentrefugen Microcentrifuge (USA), Beckman Coulter Ultracentrifuge (USA), GPE-scientific. LC-MS were an Agilent (USA). LC-MS series 6310 ion trap system equipped with electrospray ionization (ESI). Data acquisition was performed using DataAnalysis for 6300 Series Ion Trap LC/MS version 4.0 © software.

3.2.3. Methods

3.2.3.1. Preparation of buffers and solutions

Master drug stock solution of the drugs mephedrone, methcathinone and methoxetamine were prepared in deionized water at 10 times of the desired concentration (i.e. 1000 µg/ml).

The homogenizing buffer (0.1 M Tris Buffer, 10 mM EDTA and 150 mM KCl) was prepared by adding 2.922 g of EDTA and 11.1827 g of KCl to 100 mL of 1M Tris buffer saline stock solution, and then top up to 1L with deionized water. The microsomes preservation buffer (0.05 M Tris Buffer 10 mM EDTA and 20% (v/v) glycerol) was prepared by adding 0.292 g of EDTA to 5 mL of 1M Tris. Buffer stock solution, added 20 mL glycerol, and then made up to 100 mL with deionized water. The pyrophosphate buffer (0.1 M Na-pyrophosphate and 10 mM EDTA) was prepared by dissolving 2.659 g of Na-pyrophosphate and 0.292 g of EDTA in 100 mL deionized water. The final pH values were adjusted to pH 7.4 with sodium hydroxide or hydrochloric acid.

The NADPH regenerating system (30 mM G6P, 5 U/mL G6PD, 10 mM NADP and 30 mM $MgCl_2$) was prepared freshly every day before incubation.

3.2.3.2. Isolation of liver microsomes and S9 fraction

Liver microsomes were isolated following previously published protocols with minor modifications (Hill, 2003; Pigatto et al., 2011; Hofer et al., 2012). Liver was donated and obtained freshly from a local abattoir collected less than 5 minutes after slaughtering in the very early morning and directly kept ice cooled in icebox until delivery to the laboratory. Pig liver was washed with homogenizing buffer and sliced into 4 g portions. The sliced portions were further cut into small pieces using a surgical blade, broken apart with 3 volumes of ice-cold homogenizing buffer using a handheld bio-homogenizer and homogenized using a Teflon mortar and pestle. The homogenate was centrifuged for 15 min at 12,500 x g at 4°C, and the supernatant was divided into two portions, one of which was stored as S9 fraction, and the remaining fraction was further ultra-centrifuged for 45 min at 76,800 x g at 4°C. The pellet was re-suspended with 2 volumes of ice cold pyrophosphate buffer, further homogenized and ultra-centrifuged for 70 min at 76,800 x g at 4°C. The supernatant was discarded and the pellet was suspended with 3 volumes of microsomes preservation buffer and further homogenized and stored at -80°C until needed. All steps were performed over an ice bath.

3.2.3.3. Using isolated liver microsomes and S9 for metabolism studies

For each type of *in vitro* model (i.e. microsomes and S9 fraction), five 1.5-mL microcentrifuge tubes for each test compound were prepared. The five 1.5-mL microcentrifuge tubes include duplicate tubes for both 0- and 3 hours' time, and one tube for the negative control. Each tube contained 375 µL 0.1 M phosphate buffers, pH 7.4, pre-warmed to 37°C, 50 µL NADPH regeneration system and 50 µL of 10x concentration of test compound stock solution (final concentration of the drug 100 µg/mL). The mixture was then incubated in a water bath for 5 min at 37°C. For the negative control, 50 µL NADPH regeneration systems were replaced by 50 µL 0.1 M phosphate buffer. Prepared microsomes or S9 fractions were thawed and diluted to 10 mg/L and 25 µL added to each tube. Samples were mixed and incubated at 37°C in a shaking water bath. The process was terminated by adding 250 µL ice cold methanol at 0 time for two of the positive test tubes and at 3 hours for the other two positive tubes and the negative tube sample. Samples tubes were centrifuged for 10 minutes at 13,000 x g. The supernatant was then filtered through 0.45 µm filters and 5 µL injected directly onto the LC/MS system.

3.2.3.4. LC-MS detection and conditions

Chromatographic separations were carried out on Agilent Eclipse plus C_{18}, 3.5 µm, 4.6x150 mm at room temperature. The mobile phase consisted of methanol/water/formic acid (50/50/0.1, v: v: v), isocratic flow rate of 0.300 ml/min for 10 minutes. The analytes were detected in positive ionization mode (MS+). The MS instrument was tuned and calibrated according to the following conditions: Capillary voltage: 3500 V, Capillary intensity: 1 nA, Maximum acquisition time: 200 ms, Nebulizer pressure: 30 psi, Dry gas flow: 11 L/min, Dry gas temperature: 325 C.

3.3. *In vitro* studies of selected NPS using hepatocytes and analysis utilizing GC-MS

3.3.1. Materials

Mephedrone, methoxetamine, and 4-fluoromethamphetamine were purchased legally from web-based companies before any of them were declared illegal. Methcathinone was synthesized and identified in the analytical laboratory according to previously published method (DeRuiter et al., 1994). Trypan blue and phosphate buffer (pH=7) solution were from Sigma (UK).

For derivatization, BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) was from Supelco (UK).

For the study of selected NPS using pooled human suspension hepatocytes: Primary Human Hepatocytes, Cryopreserved Hepatocytes Recovery Medium (catalogue number: CM7000) and Primary Human Hepatocytes Maintenance Supplement Pack (catalogue number: CM4000) containing dexamethasone and COCKTAIL-B solution were all from Gibco (UK).

For the study of selected NPS using HepaRG: HepaRG cryopreserved cell line (catalogue number: HPRGC10), William's E Medium-no phenol red (catalogue number: A12176-01), HepaRG Thaw, Plate & General Purpose Medium Supplement (catalogue number: HPRG770), HepaRG Tox Medium Supplement (catalogue number: HPRG730), HepaRG Maintenance/Metabolism Medium Supplement (catalogue number: HPRG720) and GlutaMAX™-I Supplement (catalogue number: A12860-01) were all from Gibco (UK).

3.3.2. Equipment

Electronic balance from Sartorius (Germany), Ultrasonic bath was from Kerry (UK) and centrifuge model was Allegra X-22 from Beckman Coulter™ (UK). Sample concentrator model was DRI-BLOCK®, DB.3A from TECHNE (UK), shaking water bath was from Grant Instruments.

GC and MS components were from Perkin Elmer (USA). GC model Clarus 600 equipped with an auto sampler and MS model Clarus 600 operated with Perkin Elmer TurboMass (2008) software. Standards and samples were run on an Agilent Technologies DB-1 MS column (30 m × 0.25 mm × 0.25 µm).

3.3.3. Methods

3.3.3.1. Preparation of buffers and solutions

HepaRG Thaw, Plate, & General Purpose Medium was prepared freshly and aseptically as needed by adding 1 ml of GlutaMAX and 14 ml of HepaRG Thaw, Plate, & General Purpose Medium Supplement to 100 ml of William's Medium E.

HepaRG Tox Medium was prepared freshly and aseptically as needed by adding 1 ml of GlutaMAX and 14 ml of HepaRG™ Tox Medium Supplement to 100 ml of William's Medium E.

HepaRG Maintenance/Metabolism Medium was prepared freshly and aseptically as needed by adding 1 ml of GlutaMAX and 16 ml of HepaRG Maintenance/Metabolism Medium Supplement to 100 ml of William's Medium E

Master stock solutions of mephedrone, methcathinone, methoxetamine and fluoromethamphetamine were prepared in HepaRG™ Thaw, Plate, & General Purpose Working Medium and were at least 10 times more concentrated than the highest concentration tested (i.e. 1.6x102 mM). All stock solutions were preserved at -20C° for later use.

3.3.3.2. Preparation of cryopreserved pooled hepatocytes

Pooled Human Hepatocytes stocks were stored and maintained under liquid nitrogen (-196°C) until needed. The cryopreserved pooled hepatocytes vial was thawed and re-suspended into 10 ml media to final concentration of 1x106 cells/ml (10x106 total cells count). Cell viability was estimated to be greater than 85% using trypan blue exclusion test. Trypan blue test solution test was prepared at 0.4% in phosphate buffered solution and 0.1 ml added to 1 ml cell solution and examined under microscope

3.3.3.3. Using Cryopreserved Pooled Human hepatocytes for metabolism study:

The following final concentration of the drugs mephedrone, methcathinone, methoxetamine and 4-fluoromethamphetamine were used for metabolism studies using Pooled Human Hepatocytes (10, 20, 40, 60, 80 and 100 µM). For each one concentration, the metabolic profile was studied at the following specified times (0, 30, 60, 90 and 120 minutes). The metabolic reaction was initiated by

adding 60 µL of the prepared cell suspension and 15 µL of the 10x stock drug onto 1.5 ml Eppendorf tube and topped up with media into 150 µL (final drug concentration= 1x, final cell count > 50x103 viable cells). At the specified time, the reaction was terminated by adding 50µL 10% DMSO.

3.3.3.4. Preparation and culture of cryopreserved HepaRG™ cells

HepaRG™ Cell stocks were stored and maintained under liquid nitrogen (-196°C) until needed. HepaRG™ vial was semi-thawed in a water bath at 37° C and the cell suspension was aseptically transferred into pre-warmed 9 ml of HepaRG™ Thaw, Plate, & General Purpose Working Medium. After centrifuging cells at room temperature at 357 g for 2 minutes, the supernatant was aspirated and the pellet re-suspended in 5 ml of the HepaRG™ Thaw, Plate, & General Purpose Working Medium. The viability of the HepaRG™ was estimated to be greater than 85% using trypan blue exclusion test (see section 3.3.3.2.).

3.3.3.5. Using HepaRG for metabolism studies

The following final concentration of the drugs mephedrone, methcathinone, methoxetamine and 4-fluoromethamphetamine were used for metabolism studies using HepaRG™ (10, 20, 40, 60, 80 and 100 µM). For each one concentration, the metabolic profile was studied at the following specified times (0, 90 minute and 24 hrs). Previously prepared HepaRG cells were seeded onto central wells of collagen coated 96-well plates at a density of 50X103 cells/well in HepaRG™ Thaw, Plate, & General Purpose Working Medium (50 µL cell solution topped up to 200 µL media). After 6 hours' media was renewed with 20 µL10x master solution of each drug concentration and topped up to 200 µL HepaRG™ Tox Medium Supplement. Two wells were spared as blank which were topped up to 200 µL HepaRG™ Tox Medium Supplement media and for zero times sample, 20 µL of each drug concentration was topped up to 200 µL HepaRG™ Tox Medium Supplement media.

3.3.3.6. Preparation of samples produced from using Pooled Human Hepatocytes for metabolism studies for GC-MS analysis

The final 200 µL resultant aqueous mixture was extracted using with 400µL ethyl acetate, and then centrifuged at 3500 rpm for 10 minutes. Organic layer was moved into a clean glass vial via a pipette

and then evaporated to dryness using sample concentrator under streaming N_2, reconstituted with 50µL acetonitrile and then injected onto the GC-MS system under the specified conditions.

After initial runs for the prepared samples utilizing GC-MS, samples were derivatized with BSTFA. BSTFA was added in excess – 100 µL - to the sample and the mixture warmed in water bath at 50 C◦ for 10 minutes to enhance the derivatization reaction. The derivatized sample was then injected into the GC-MS system under the specified conditions.

3.3.3.7. GC-MS detection and conditions

Oven temperature was an initial 4-min plateau at 150C˚ increasing at 25C˚/min to a final temperature of 350C˚. Programmable injector port temperature was held at 250C˚ for 4 min and then increased to 300C˚ at 50C˚/min. The final temperature was held for 7 min to correlate with the total run time of 12 minutes. The carrier gas was helium at 1 ml/min and the injection volume was 1 µl. The transfer line temperature was held at 280 C◦. Positive ionization was achieved using an Electron Impact (EI+) source at 200C◦ with electron energy of 70 eV and the multiplier was set to 300 V. The peaks were observed in total ion count (TIC) mode for 9 minutes after 3 min solvent delay giving a total run time of 12 minutes. The scan range was 45–320 m/z with scan time of 0.5 second and an inter-scan delay of 0.01 seconds.

3.3.3.8. Using HepaRG™ for MTT reduction studies – cytotoxicity

Previously prepared HepaRGTM cells were seeded onto central wells of collagen coated 96-well plates at a density of 100X103 cells/well in HepaRG™ Thaw, Plate, & General Purpose Working Medium (50 µL cell solution topped up to 200 µL media). Two wells were spared as blank control, and the peripheral wells were filled with sterile water to prevent media evaporation. The 96-well plate was incubated at 37C° in a humidified atmosphere with 5%/95% CO_2/ambient atmosphere and 100% relative humidity for cell adhesion. After 6 h the medium was renewed, and on day 1 and day 4 the medium was replaced with HepaRG™ Tox medium and keep incubated under same conditions, while observing cell morphology on time of replacing media, the MTT reduction assay experiments were carried on day 7 with freshly prepared test drugs. On day 7 after cell seeding, the HepaRG™

Tox medium was renewed and the cells were incubated for 48 h with the different concentrations of the drugs mephedrone, methcathinone, methoxetamine and fluoromethamphetamine at 37C°, in a humidified atmosphere with 5%/95% CO_2/ambient atmosphere and 100% relative humidity. Those drugs concentrations were freshly prepared by from master stock concentration in HepaRG™ Tox medium. Each individual plate included two replicates blank controls (just media) and two replicates of negative control (full cell and media with no test agents). After the 48 h incubation period, the incubation medium was aspirated, and the attached cells were washed one time, followed by the addition of fresh HepaRG™ Tox medium containing 0.5 mg/L MTT. The cells were re-incubated at 37C° in a humidified atmosphere with 5%/95% CO_2/ambient atmosphere and 100% relative humidity for 3 h. After aspirating the medium, formed insoluble crystals were dissolved in 100% dimethyl sulfoxide (DMSO). A multi-well plate reader was used to run the spectrophotometric analysis at 570 nm wavelength.

For each drug, a broad range (4.0x10-2 – 1.6x101 mM) was tested, and each was done in three independent experiments. The mean absorbance of the triplicate experiments for each drug concentration on each occasion was expressed as a percentage of the mean absorbance of the control wells.

Chapter 4 Results and discussion

4.1. Analysis of mephedrone and other selected drugs of abuse utilizing HPLC after extraction from biological samples

Mephedrone is a synthetic cathinone derivative that started to appear online and become available in the market in 2007, Until April 2010, when there was a governmental decision in UK of classifying mephedrone as class B, so its use, supply or possession was banned (Torrance and Cooper, 2010). There was no available valid quantitative HPLC-DAD analytical method for its analysis in biological fluids, at the date research started in 2009, and also there was little data available about its clinical or toxicological profile. Therefore, the aim of our research was to develop and validate a quantitative analytical method for easy and rapid analysis of mephedrone, alone or in combination with other drugs of abuse such as methcathinone, utilizing HPLC-DAD after extraction from biological fluids. HPLC-DAD is one of the more common analytical instrumentation in most forensic and analytical laboratories, and reversed phase chromatography is the most common form used generally in liquid chromatography.

For this part of the work the stationary phase chosen for analysis is, C_{18}, 5 μm, 4.6 x 150 mm columns. This type of column contains a chemically modified silica backbone of relatively of low polarity in comparison to the polarity of the most type of solvents used in liquid chromatography. The analysis of analytes of interest was performed by the use of an isocratic mobile phase consisting of a mixture of water and acetonitrile adjusted to acidic pH values Acetonitrile, compared to methanol, is more efficient at low pH values, and has a lower viscosity. Considering the reported pKa value of the analytes of interest of 8.4-9.5 for mephedrone and methyl cathinones in general (Gibbons and Zloh, 2010), the mobile phase was adjusted to low pH values, as that would improve the shape of the peaks when analysing basic drugs. Buffering the mobile phase is very important, as this would assure reproducible retention peaks. These stationary and mobile phases are compatible with reversed phase chromatography and were expected to behave well for the analysis of the selected drugs that are mainly basic in nature (Neue et al., 2001; USP-NF, 2007b; Snyder et al., 2009; Gibbons and Zloh, 2010; McCalley, 2010).

Acetamidophenol, nicotinamide, or aniline were used as internal standard in this part of the current work since they are expected to have nearly similar physicochemical properties; being basic in nature due to the presence of amine functional groups and non-polar due to the prevalence of C-C and C-H bonds (Figure 16). Similar studies utilizing HPLC for the analysis of the related amphetamines used one of them as internal standard (Hutchaleelaha et al., 1994; McAvoy et al., 1999).

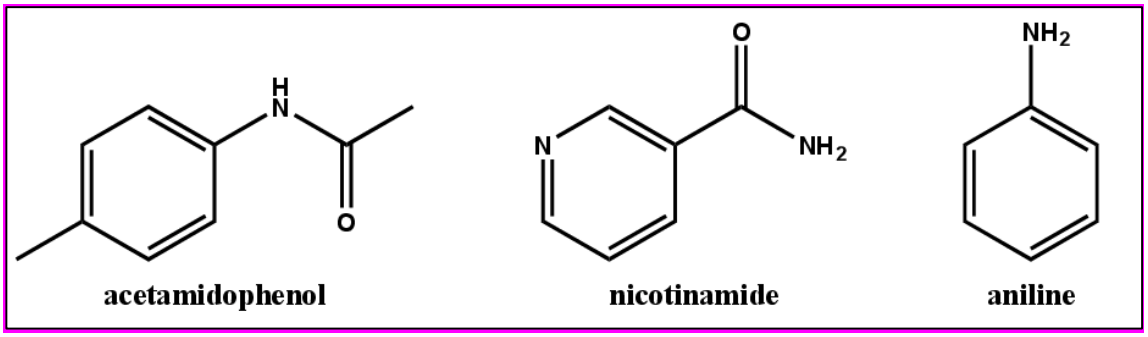


Figure 16: Chemical structure of internal standards used in HPLC-DAD analysis

As diode array detector (DAD) is one of the commonly available detector types in forensic laboratories with its ability to provide more spectral information than the fixed UV detector, it was used in this part of the work. Scanning was performed to determine the wavelength of maximum absorbance for the analytes, and detections were carried out at each drug's maximum absorbance wavelength to improve method sensitivity.

4.1.1. Developing HPLC method(I) for the analysis of mephedrone

For qualitative and quantitative analysis, chromatograms produced after injection of mephedrone standards were identified and observed. Blank samples showed base line chromatograms, while different concentration of mephedrone standard chromatograms showed the appearance of one peak with retention time of ~ 6.75 minutes where the peak height was proportional to the standard concentration, which in conclusion was indicative that these peaks correspond to the drug mephedrone. The peaks showed tailing which indicates a type of interaction between the mobile phase and stationary phase, which may need further optimization for the type and constituents of the mobile phase. Buffering the mobile phase will control the ionization of the analyte and minimize the tailing of peaks and insure reproducible retention times (Ardrey, 2003).

Diode array detectors (DAD) are very important tool for the identification of compounds, where the detector records the absorbance over a range of up to 750 nm covering the UV range (190-380 nm) and allowing acquisition of a spectral data profile including the wavelength of maximum absorbance. However, some drugs of the same class may show similar UV spectra, which need other qualifiers for the identification of the analyte. For mephedrone, spectral scanning of the identified peak was carried out via DAD at 220-400 nm, and the wavelength of maximum absorbance, in combination with retention time of mephedrone was used as qualifiers for the identification of mephedrone. The spectral scanning has many useful applications. Peak purity, for example, can be determined form the study of the absorbance of one analyte at different wavelength. In this part of the work, maximum absorbance of mephedrone was determined to be 262 nm, which will be used later for qualitative and quantitative analysis (Figure 17).

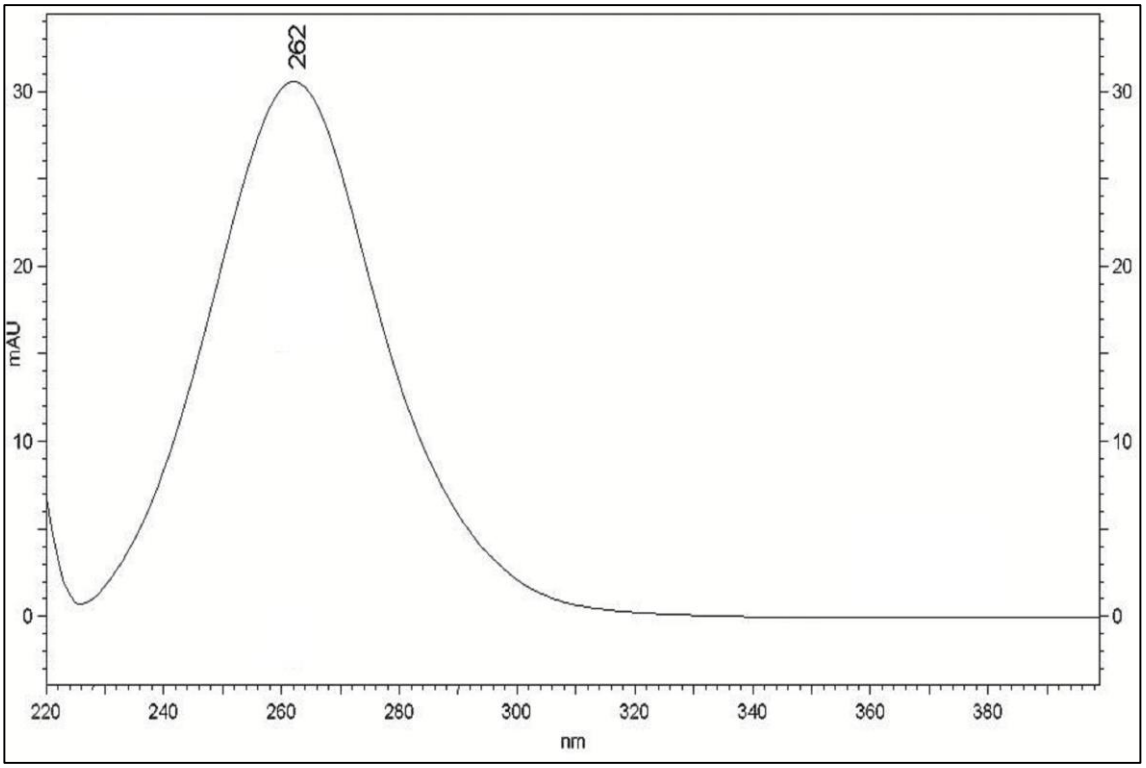


Figure 17: Spectral scanning of mephedrone via DAD (220 nm – 400nm)

The observed maximum absorbance of the mephedrone is comparable to the reported maximum absorbance for mephedrone hydrochloride and mephedrone hydro bromide of 263.5 for standards either prepared in deionised water or 0.1 M aqueous hydrochloric acid, and of 259.5 for standards

either prepared in absolute ethanol or 0.1 M aqueous sodium hydroxide (Santali et al., 2011). Retention time and the wavelength of maximum absorbance were used as qualifiers for the identification and quantification of the drug mephedrone. The integration of peak areas was carried out at the specified wavelength to increase sensitivity of the method. Increasing the standard concentration was reflected on peak areas, and the developed HPLC method showed to be linear over the specified range with R2= 0.989 (Figure 18)

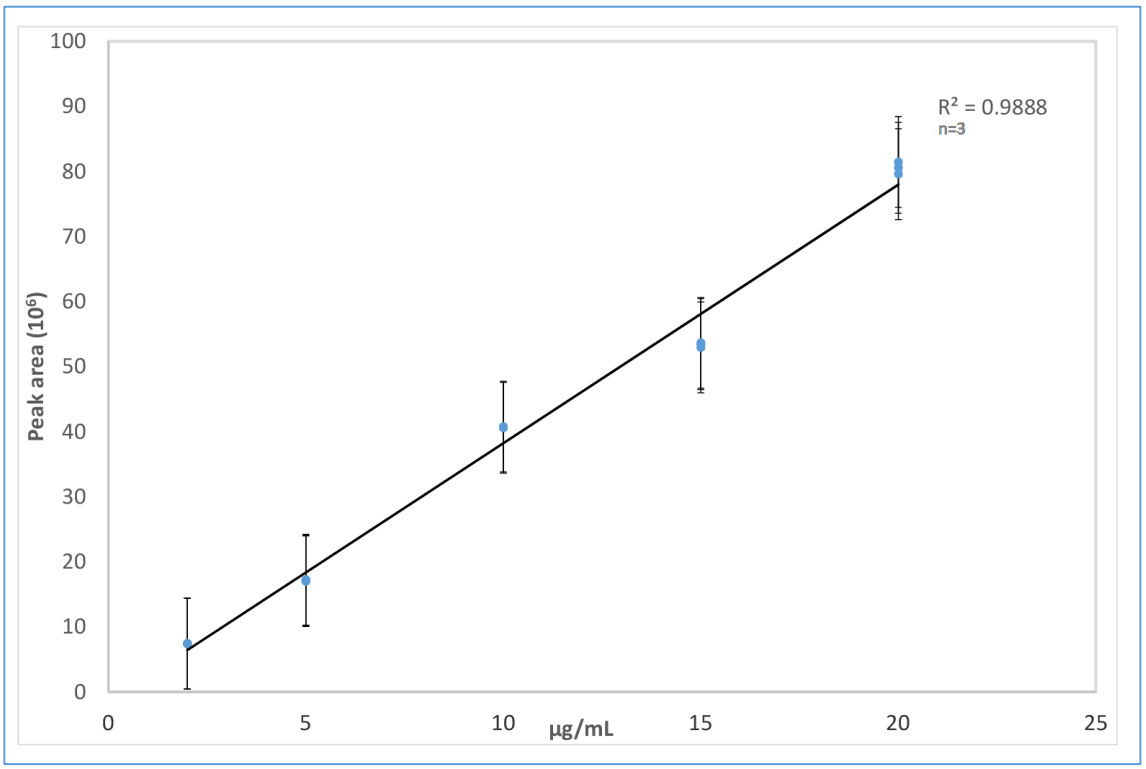


Figure 18: calibration curve of mephedrone (2-20 µg/ml) in water.

Mephedrone was prepared in mobile phase aiming to study the effect of different solutions on the retention time, peak shape and maximum absorbance of the drug. Using different solution other than the mobile phase may interfere with the equilibrium and interaction between mobile and stationary phase. However, there is no specific recommendation in the literature about specific type of solution to prepare samples, though it is preferable if applicable to use the mobile phase (Keunchkarian et al., 2006; Snyder et al., 2009). There was no significant difference between retention time, peak shape, UV spectrum, wavelength of maximum absorbance or the integrated areas of corresponding concentration, prepared in either water or mobile phase. However, there was minor improvement in the linearity of the HPLC method with R2=0.999. Peak areas were integrated at the wavelength of

maximum absorbance which was determined previously to be 262 nm. The drug mephedrone identity was confirmed by previously determined retention time of 6.75 minutes (Figure 19) and the wavelength of maximum absorbance at 262 nm (Figure 20).

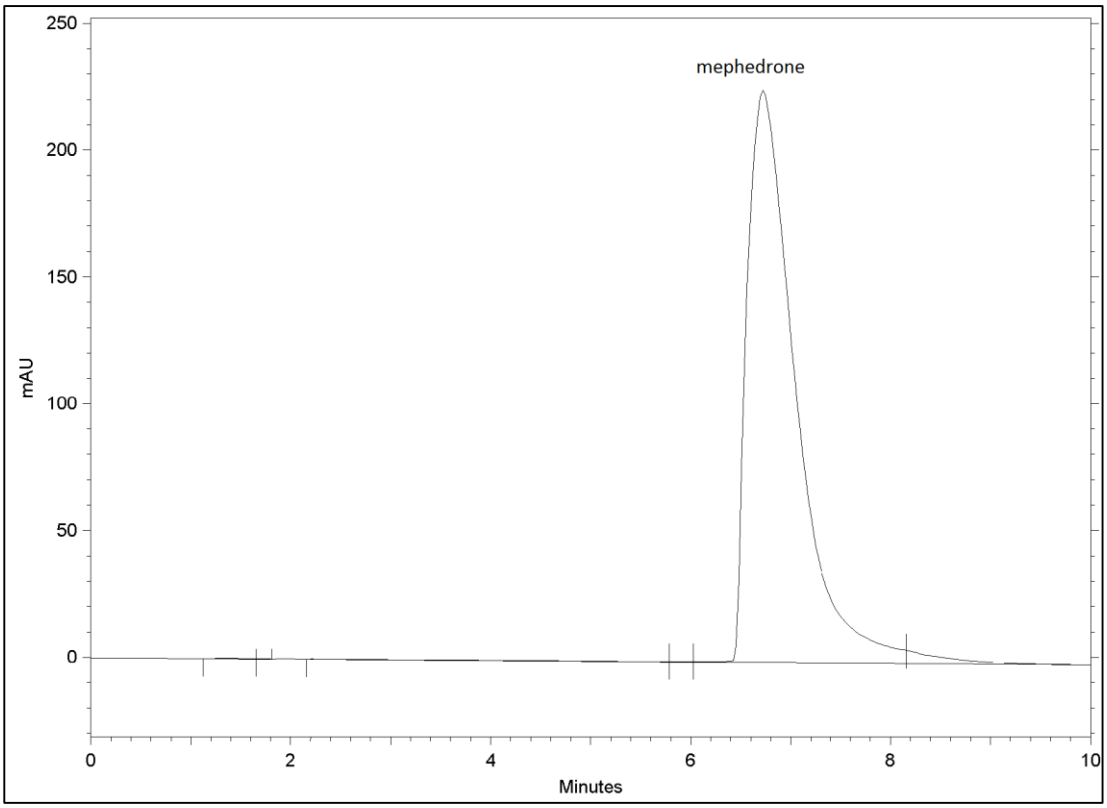


Figure 19: representative HPLC chromatogram at 10 µg/ml of mephedrone in mobile phase by DAD (200-400nm)

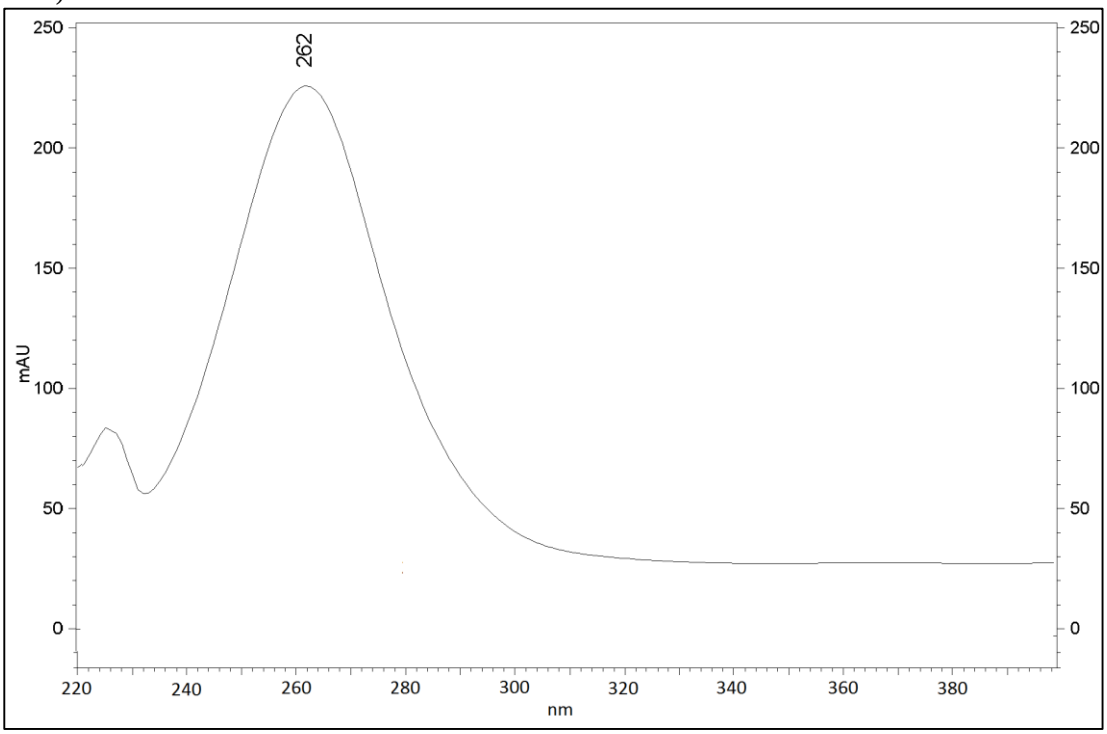


Figure 20: representative UV spectrum and wavelength of maximum absorbance at 10 µg/ml of mephedrone in mobile phase via DAD (220-400nm)

Ruta et.al investigated the effect of sample diluents on peak shape, specifically for hydrophilic liquid chromatography, concluding that pure acetonitrile is the best solvent for obtaining the best peak shape. However, in case of any issues of using pure acetonitrile, solubility for example, isopropyl alcohol only or as a mixture with acetonitrile is a valid alternative (Ruta et al., 2010).

As discussed by Keunchkarian et.al, sample and standard prepared in different solvents other than mobile phase is one of the possible reasons for atypical peak-shapes, affecting mainly the early eluting analytes. They concluded that hydrodynamic instability and interference of the sample solvent plug with the equilibria between the mobile and stationary phase could produce distortion affecting the peak shape and concentration profile when running analysis using reversed phase chromatography. In their recommendation, they suggest many solutions to minimize the effect of sample solvent/mobile phase viscosity mismatch (Keunchkarian et al., 2006).

In HPLC, the eluting power of a solvent is measured by its ability to move compound over the adsorbent surface. In general, eluting power is related to polarity, where more polar compounds have stronger eluting power. The effect of sample solvent on the analysis is more evident when introducing the sample in a solvent stronger than the mobile phase. In fact, introduction of the sample in weaker solvent is possible without any effect on the peaks (Snyder et al., 2009). This may explain that changing using water as sample solvent was having no effect on peak shape or integrated peak areas.

Compared to later findings by Santali et.al, where mephedrone showed difference in the maximum absorbance in different types of solutions, in the current work, there was no difference in maximum absorbance in samples prepared either in water or in mobile phase, which is mixture of water and acetonitrile. However, the determined maximum absorbance by Santali et al. was confirmative to the one observed in the current study. In their study, Santali e.al determined the maximum absorbance of mephedrone to be 259.5 and 263.5 compared to 262 nm in our work (Santali et al., 2011).

The analytical method was optimised by adjusting either the type of the internal standard or flow rate. The aim of the optimization was to achieve sufficient resolution between the peaks of the

internal standard and that of mephedrone with the shortest run time. Other HPLC conditions were kept the same. Separation of mephedrone and the internal standard in less than 10 minutes with good resolution between the peaks was satisfactorily achieved for the current part of the work.

Adjusting the flow rate will have an inversely proportional effect on the total run time, as decreasing the flow rate will increase the total run time. In general, the increase in the total run time will allow analytes that elute in a narrow window to separate at wider intervals improving the resolution. Long run time is disadvantageous and time consuming. By increasing the flow rate, total run time will shorten. However, this will negatively affect the resolution between the peaks. In case of presence of different analytes with different polarities, using gradient elution may overcome this issue as dynamic change of the composition of the mobile phase will separate analytes of different polarities within shorter time compared to using isocratic elution

In the first set of experiments, acetamidophenol was selected as an internal standard. Using isocratic elution at a flow rate of 1 ml/min achieved the goal of less than 10 minutes' analysis time, but there was insufficient resolution between the peaks, as the peaks appeared overlapped. The identity of the first appearing peak was confirmed to be mephedrone as increasing the concentration of the mephedrone standard was reflected by increase in peak height. In addition, the maximum absorbance determined by tracing of the UV spectral scanning of the peak and wavelength of maximum absorbance. UV spectrum and wavelength of maximum absorbance of mephedrone were similar to those previously observed. Using acetamidophenol as internal standard at a flow rate of 1 ml/min, there was insufficient resolution between the peaks. Flow rate was adjusted in two separate experiments to 0.2 ml/min. and 0.5 ml/min while keeping the mobile phase mixture the same. Both trials did not achieve the goal of a less than 10-minute analysis time. However, the resolution between the peaks improved in both cases at flow rates of 0.5 and 0.2 ml/min, with the best resolution in the latter. The identity of the first appearing peak was confirmed to be mephedrone as increasing the concentration of mephedrone standard was reflected in an increase in peak height. The maximum absorbance determined using spectral features was also studied and used in the confirmation (Figure 21).

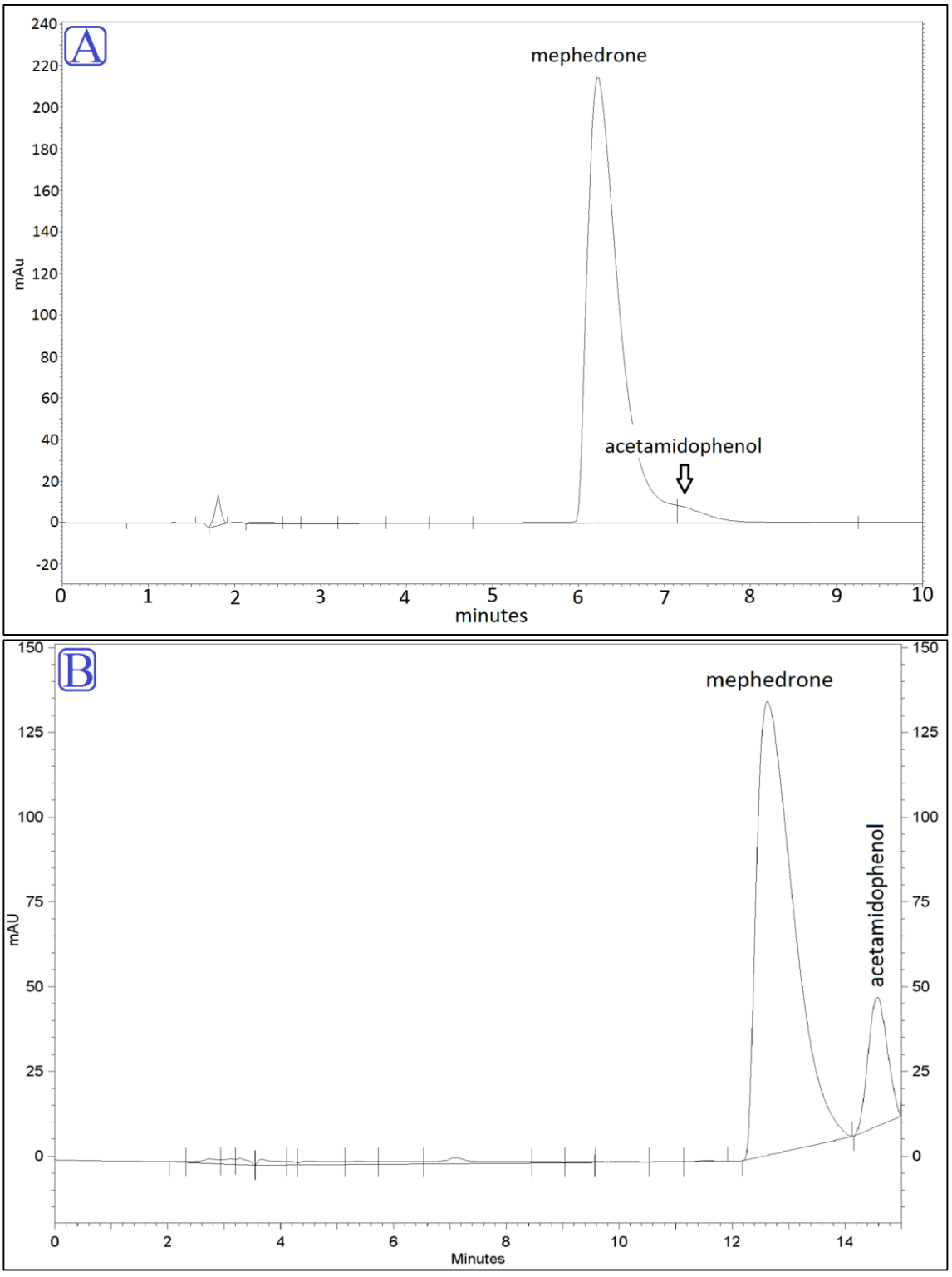


Figure 21…. Continued

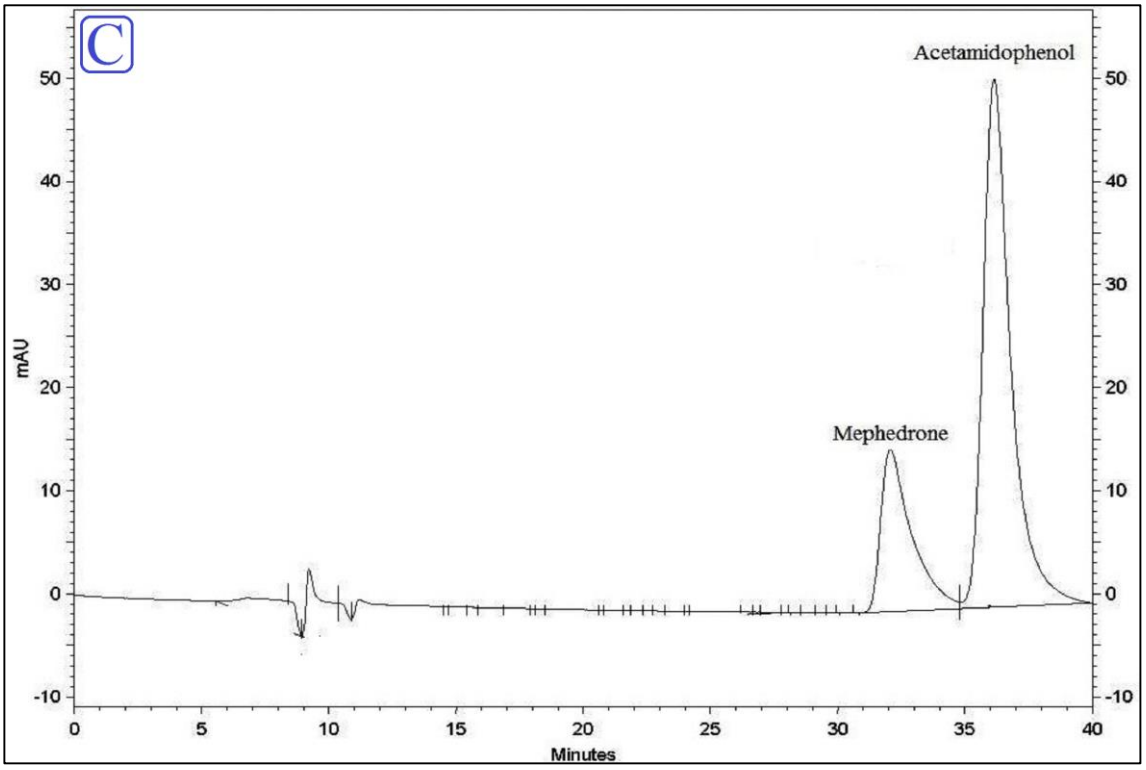


Figure 21: Representative HPLC profile of the separation of mephedrone standard in presence of the internal standard acetamidophenol adjusting flow. Optimizing HPLC method (I) for the analysis of mephedrone.
At flow rate of A = 1ml/min, B =0.5 ml/minute and C =0.2ml/min

In the second set of experiments, nicotinamide was selected as an internal standard instead of acetamidophenol. Keeping the mobile phase mixture, the same of H_2O: ACN: Acetic acid (85:10:2.5) at flow rate of 1 ml/min achieved the goal of a less than 10-minute analysis time and satisfactory resolution between the peak. Depending on the results of the optimization steps, the aim of best resolution and shortest analysis time was achieved by using nicotinamide as an internal standard and adjusting the flow rate to 1 ml/min. (Figure 22).

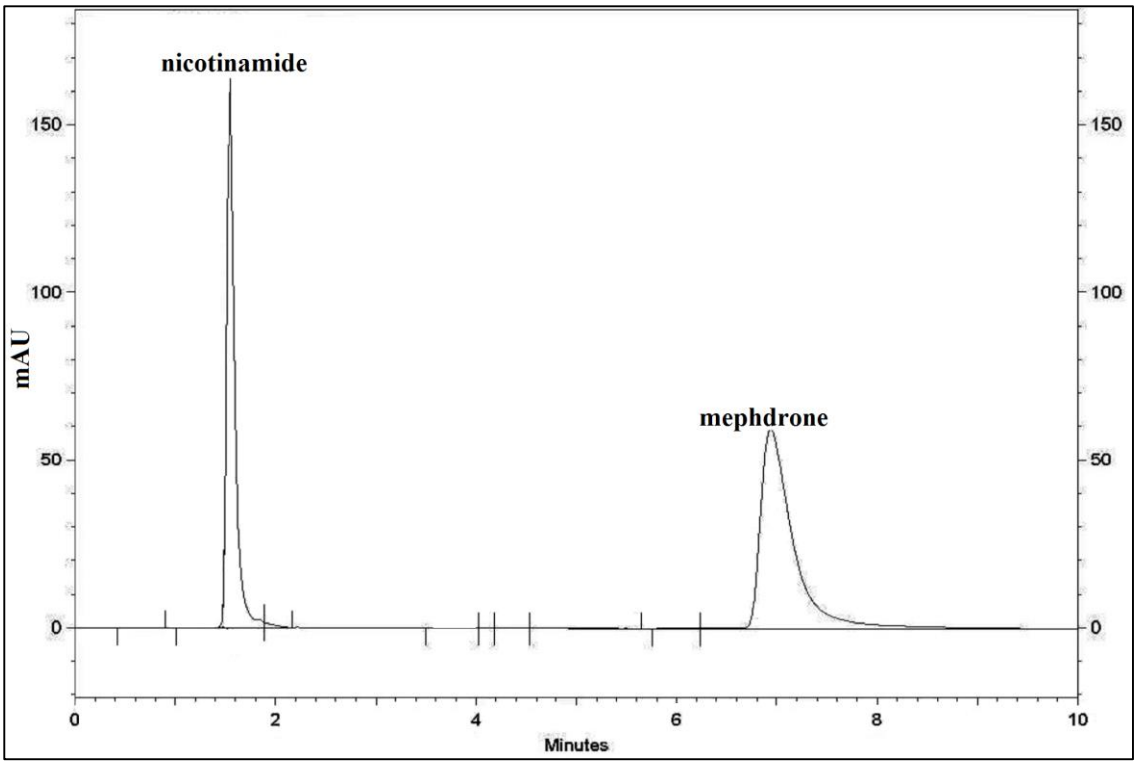


Figure 22: representative HPLC profile of the separation of mephedrone standard in presence of the internal standard nicotinamide adjusting flow. Optimizing HPLC method (I) for the analysis of mephedrone

In this part of the work, the interest was of one analyte, mephedrone, and the issue of low resolution with the aim of short run time was resolved by the selection of an internal standard that elutes very early compared with the analyte of interest, mephedrone. Summary of the results are presented in Table 9.

Table 9: Summary of the results obtained optimizing the developed HPLC-DAD method (I) for the analysis of mephedrone

Chromatography conditions	FR[1]	I.S[2]	rt1[3]	rt2[4]	comments
Mobile phase: H_2O: ACN: Acetic Acid (85:10:2.5), Column: Phenomenex® (C_{18}, 150 ×4.6 mm, 5µm), Isocratic elution, ambient temperature and DAD spectral scan range: 200-400 nm	1	Acetamidophenol	6.6	7.1	- Total run time < 10min - insufficient resolution
	0.5	Acetamidophenol	12.6	14.6	- Total run time > 10 min - insufficient resolution
	0.2	Acetamidophenol	32.0	36.1	- Total run time > 10 min - Better resolution
	1	Nicotinamide	6.7	1.5	- Total run time < 10 min - Satisfactory resolution

1: mobile phase flow rate (ml/min) 2: internal standard 3: retention time of mephedrone (minutes) 4: retention time of internal standard (minutes)

4.1.2. Experimentation of LLE of mephedrone from whole blood samples followed by analysis applying the optimized HPLC-DAD method (I)

The previously optimized HPLC method I (section 4.1.1. , p. 85) was applied after LLE of mephedrone from whole blood samples. The experimentation steps involved either modifying pH of the sample or type of the extraction solvent. The efficacy of the LLE method was observed by calculation of the percent of recovery.

For LLE, adjusting pH, the extraction liquid to matrix ratio, in addition to the type of solvent are the major factors affecting the efficiency of the extraction process The type and pH of buffer solution will control the ionization of the analytes and partitioning between the solvents. Increasing the ratio of the extracting solvent to matrix, in favour of the extracting solvent will increase the chance of transferring fraction of the analytes from their matrix. The type of solvent and its physicochemical properties including polarity, miscibility and boiling points are important factors controlling the efficacy of LLE process.

The efficiency of the LLE process was assessed primarily by calculating recovery percentages (extraction fraction). Recovery percentages were calculated as the ratio of peak area after extraction against the peak area of the corresponding standard concentration Performing LLE of mephedrone from unbuffered blood samples, it was possible to extract less than 10% of the known concentration of the spiked mephedrone. When modifying sample pH before extraction with a buffer solution of pH 3, less than 4% of mephedrone was extracted. Modifying the sample with a neutral buffer solution (pH 7), it was possible to extract relatively similar fraction mephedrone of less than 10%. Higher recovery percent at the range of 10-35% were obtained by modifying sample pH before extraction with buffer solution of pH 10.

By modification of extraction solvent contents and using buffer solution of pH 10, higher recovery percent were obtained using ethyl acetate as an extraction solvent– 10-35% compared to less than 10% when using hexane as extraction solvent (Table 10)

Table 10: Summary of the results of obtained optimizing LLE of mephedrone from blood

Extraction solvent	Modifying buffer pH	Comments
Ethyl acetate	Unbuffered	Extraction fraction <10 %
Ethyl acetate	3	Extraction fraction <4%
Ethyl acetate	7	Extraction fraction <10 %
Ethyl acetate	10	Extraction fraction 10 - 35%
Hexane	10	Extraction fraction <10%

The effect of pH and the maximum recovery percent obtained with the modification of pH=10 could be explained by the expected pKa values of 8.4-9.5 for mephedrone and methyl cathinones in general (Gibbons and Zloh, 2010). In low pH values, basic drugs are in their ionized state and will preferably stay into the aqueous phase, which negatively affects the recovery percentage. At high pH values, typically above the drugs pKa values, basic drugs are in their unionized state and will preferably partition into the organic phase and consequently higher recovery percentages would be obtained. Basic drugs are best extracted into organic solvent in pH values 1-2 pH units more than their pKa values. Concluded from the above discussion, LLE of buffered samples with buffer solution of pH 10 were the optimum method parameters.

4.1.3. Validation of the optimized method of LLE of mephedrone from blood samples followed by analysis utilizing HPLC-DAD

Linearity of the HPLC analytical method before extraction was assessed by preparing five points of mephedrone standards (range 2-10 µg/mL) with added internal standard nicotinamide (100 µL of 20 mg/dl) into 1 ml drug standards. Each standard injected onto HPLC in triplicate and each peak area of the analyte mephedrone and the internal standard nicotinamide was integrated and the peak area ratio (PAR) calculated and plotted against corresponding concentrations. The analytical method before extraction was shown to be linear wit R2=0.9951.

Linearity of the HPLC analytical method before extraction was assessed by preparing five points of pre-spiked whole blood samples with known concentrations of the drug mephedrone (range 2-10 µg/mL) with added internal standard nicotinamide (100 µL of 20 mg/dl) into 1 ml blood. Each standard was injected onto the HPLC system in triplicate, and each peak area of the analyte mephedrone and the internal standard nicotinamide was integrated and the peak area ratio (PAR)

calculated and plotted against corresponding concentrations. The analytical method (after extraction) yielded a plot with R2 value > 0.99 (Figure 23). Relative standard deviation (RSD %) at each point was less than 2%. The specified range was comparable to the reported fatal plasma concentration of the drug mephedrone in the range of 2 µg/ml, (Torrance and Cooper, 2010; Green et al., 2014).

For the assessment of repeatability (intraday precision), six standards of the drug mephedrone at 100% of the maximum range (i.e. 10 µg/mL) were prepared, extracted the same day following the optimized LLE protocol and injected onto the HPLC-DAD under the specified condition. LLE extraction process showed repeatability within the same day with RSD% = 4.36%. For the assessment of intermediate precision (interday precision) method was assessed over different days at drug concentration 10 µg/mL. LLE extraction process showed intermediate precision over different days with RSD% = 4.77%. Accuracy, which is reported as percent recovery (ICH, 2005), was low and variable. Percent recovery values were calculated as ratio of peak areas after extraction against the corresponding peak area before extraction for each drug concentration and for the internal standard. Recovery fraction for mephedrone ranged between 28 – 37% and 16-23% for the internal standard.

Limit of detection and limit of quantification were calculated mathematically applying the previously mentioned equation in section 3.1.3.3. Peak areas were applied instead of peak heights. The minimum tested sample concentration with acceptable RSD% was 2 µg/mL. Average background noise was calculated 480. The calculated LOD and LOQ were 0.025 and 0.083 µg/mL, respectively.

Emulsion formation was the major problem faced, affecting both repeatability and recovery percent. Summary of the results of the validation parameters are presented in Table 11.

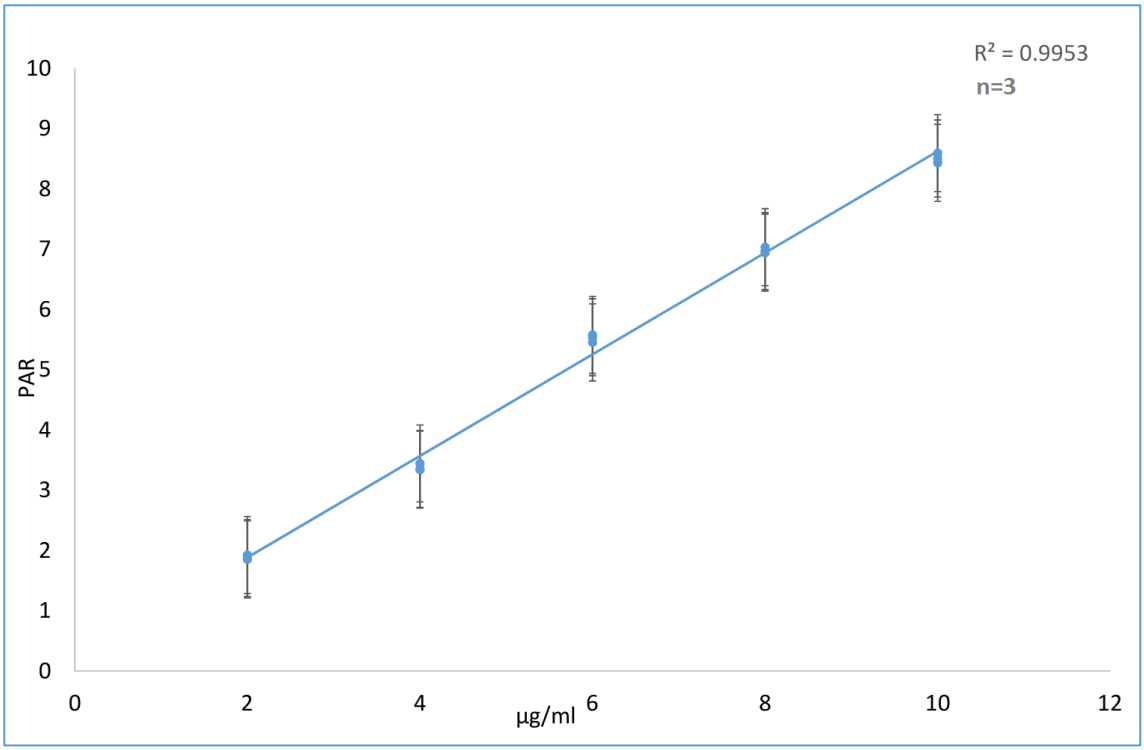


Figure 23: Calibration curve of method of analysis of mephedrone utilizing HPLC-DAD after LLE from whole blood samples

Table 11: summary of the results for the validation of the optimized method of LLE of mephedrone from blood samples followed by analysis utilizing HPLC-DAD

Linearity (R^2)		**Precision (RSD%)**		**Accuracy (recovery percent)**		**Limits (µg/mL)**	
Before LLE	0.9951	Repeatability (intraday precision)	4.36%	mephedrone	28-37%	LOD	0.025
After LLE	0.9953	Intermediate precision (interday precision)	4.77%	nicotinamide	16-23%	LOQ	0.082

4.1.4. Applying and validation of the optimized LLE followed by HPLC-DAD for simultaneous analysis of mephedrone, amphetamine, caffeine, ketamine, and codeine from blood sample

Following the LLE protocol in section 3.1.3.4. , as noticed earlier, the results showed impairment for the extraction process for all drugs including the internal standard at all concentrations reflected by variable and low recovery values. Emulsion formation was the major problem faced, affecting both repeatability and recovery percent. Calibration curves were produced by injecting standard concentrations of the analytes mixture onto the HPLC in the presence of internal standard. For quantitative analysis, peaks were integrated at the wavelength of maximum absorbance of

mephedrone and peak area ratios plotted against the corresponding concentration. The produced calibration curves showed linearity for all analytes with R2> 0.99 for all analytes.

Linearity of the method (after extraction) was assessed by plotting peak area ratios against corresponding concentration. The analytical method was shown to be linear over the specified range for each analyte with R2 > 0.99 for all analytes. For the assessment of precision, peak area ratios variations were calculated for each concentration.

Repeatability (intraday precision) was assessed by computing RSD of triplicates analysed the same day and intermediate precision (interday precision) was assessed by computing RSD values for samples analysed over different days. For repeatability, RSD% values ranged between 0.17% and 3.47%, and for intermediate precision RSD% values ranged between 1.17% and 4.78%. The recovery values were variable and ranged between 9% and 90%. Lowest recovery values were obtained for mephedrone which ranged between 9-30% (average 23%) and recovery values of other analytes were relatively comparable and ranged between 20-90 % (average 55-67%).

Emulsion formation was the major problem faced also and affected both repeatability and recovery percent, as demonstrated by variable and low recovery values. Summary of the results of the validation parameters are presented in Table 12.

Table 12: summary the validation results for applying the optimized LLE followed by HPLC-DAD for simultaneous analysis of mephedrone, amphetamine, caffeine, ketamine, and codeine from blood sample

	mephedrone	**amphetamine**	**caffeine**	**ketamine**	**codeine**
Range (µg/ml)	0.2 – 5	0.2-5	20-60	10-50	10-50
calibration (R^2)	>0.99	>0.99	>0.99	>0.99	>0.99
Linearity – after LLE (R^2)	>0.99	>0.99	>0.99	>0.99	>0.99
repeatability	0.28-2.88%	0.17-2.69%	0.22-2.11%	0.47-3.47%	0.20-2.33%
Intermediate precision	2.10-4.07%	1.51-478%	20.6-3.15%	1.17-4.32%	2.06-3.67%
Recovery%	9-30% (23%)	20-79% (55%)	23-88% (61%)	25-90% (67%)	25-90%(63%)

When the validation parameters of the analytical method when applied for the analysis of different drugs mixture are compared to the validation parameters when applying the same analytical method for the analysis of mephedrone are compared, values were relatively comparable. The recovery

percentage of the drug mephedrone were relatively similar and were below 37% in both cases. However, the recovery percentages of other drugs were relatively higher and were above 55% for all drugs other than mephedrone. In both applications of the analytical method, recovery percentage were variable and ranged between 9-90%. Precision values resembled by RSD% were relatively comparable.

Though emulsion formation was the major problem faced, it was possible to recover up to 90% of ketamine and codeine for example, while the maximum recovered mephedrone was 37%. This is suggestive that it is not only the emulsion formation that impaired the recovery of mephedrone. Another possible factor that affected the efficacy of the extraction process is the type of the extraction solvent (ethyl acetate) and the preference of mephedrone to partition into it.

4.1.5. Optimization of the developed HPLC method for the simultaneous analysis of mephedrone and methcathinone (method II)

As discussed earlier, the retention of ionisable basic drugs in the column during HPLC analysis, is extremely dependent on the type and ratio of organic modifier and on pH of the mobile phase. Buffering the mobile phase is very important to ensure reproducible retention peaks, due to the expected basic nature of the drugs mephedrone and methcathinone. In this optimization, the mobile phase was pH buffered by acetate buffer of pH 4.1.

As the nicotinamide retention time was very early (1.5 minutes), and most of the interferences were appearing early in the chromatogram, it was preferable to use another internal standard. Aniline was selected as new internal standard as it is expected to have nearly similar chemical properties and to behave in a similar way to the analytes of interest when extracted, being basic in nature due to the presence of amine functional groups and non-polar due to the prevalence of C-C and C-H bonds (see Figure 16,p 85).

Adjusting the pH of mobile phase with acetate buffer, good resolution between the peaks and less than 10 minutes run time has been obtained using aniline as internal standard and peak shapes improved when using low pH buffered mobile phase. Adjusting pH of the mobile phase in reversed

phase chromatography will not only improve the peak shapes, but also the selectivity of the analysis (Neue et al., 2001; McCalley, 2010).

Compared to previous chromatograms using unbuffered mobile phase (e.g. see Figure 19 , p. 88), there was an improvement in the peak shapes. Aniline was used as internal standard in this optimized HPLC method, and it was eluted between the two analytes of interest. Satisfactory resolution between the peaks was obtained. The risk of early appearing peaks observed previously with nicotinamide and the possible interference with solvent fronts peaks was overcome improving the qualitative and quantitative analysis (Figure 24).

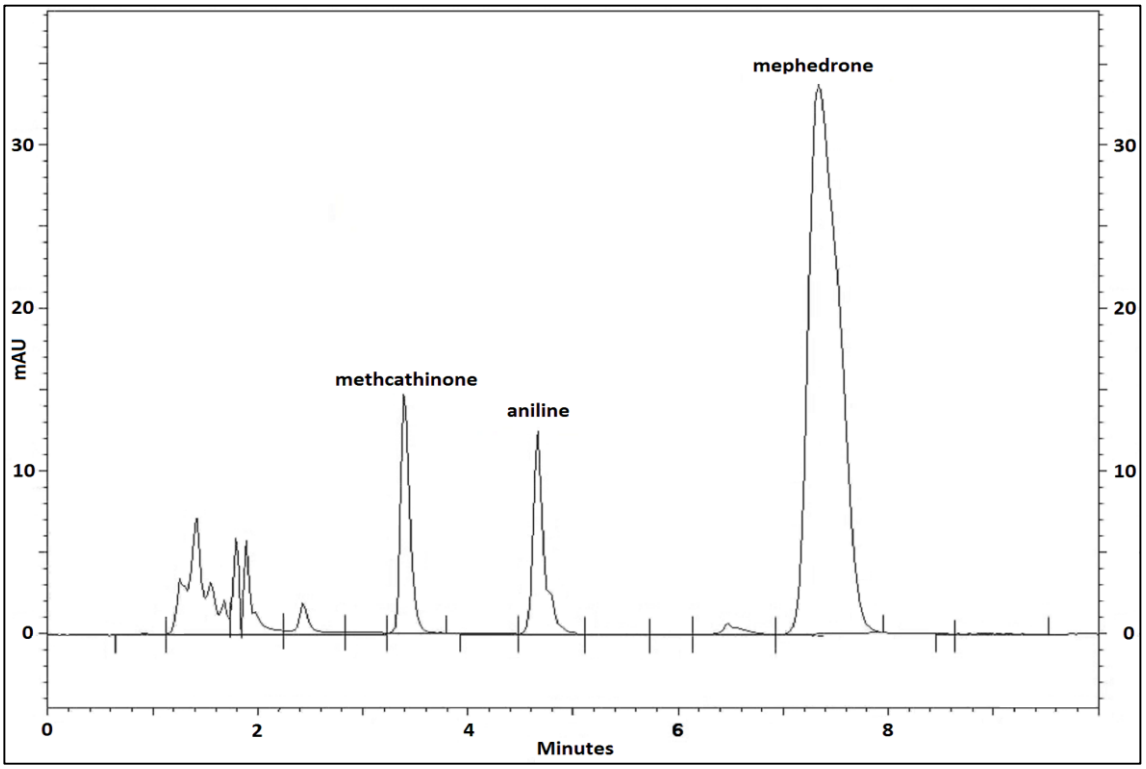


Figure 24: representative HPLC profile of the separation of mephedrone, methcathinone and internal standard (aniline) optimizing HPLC method (II)

Earlier in this thesis, the wavelength of maximum absorbance for mephedrone was determined to be 262 nm (see Figure 17, p 86). The wavelength of maximum absorbance for methcathinone was determined to be 250 nm (Figure 25) as an optimum in this separation. The spectral scan for the

peaks of aniline showed two maximal absorbance's at 278 and 237 nm (Figure 26) and the wavelength of 237 was used for quantitative analysis as an optimum in this separation.

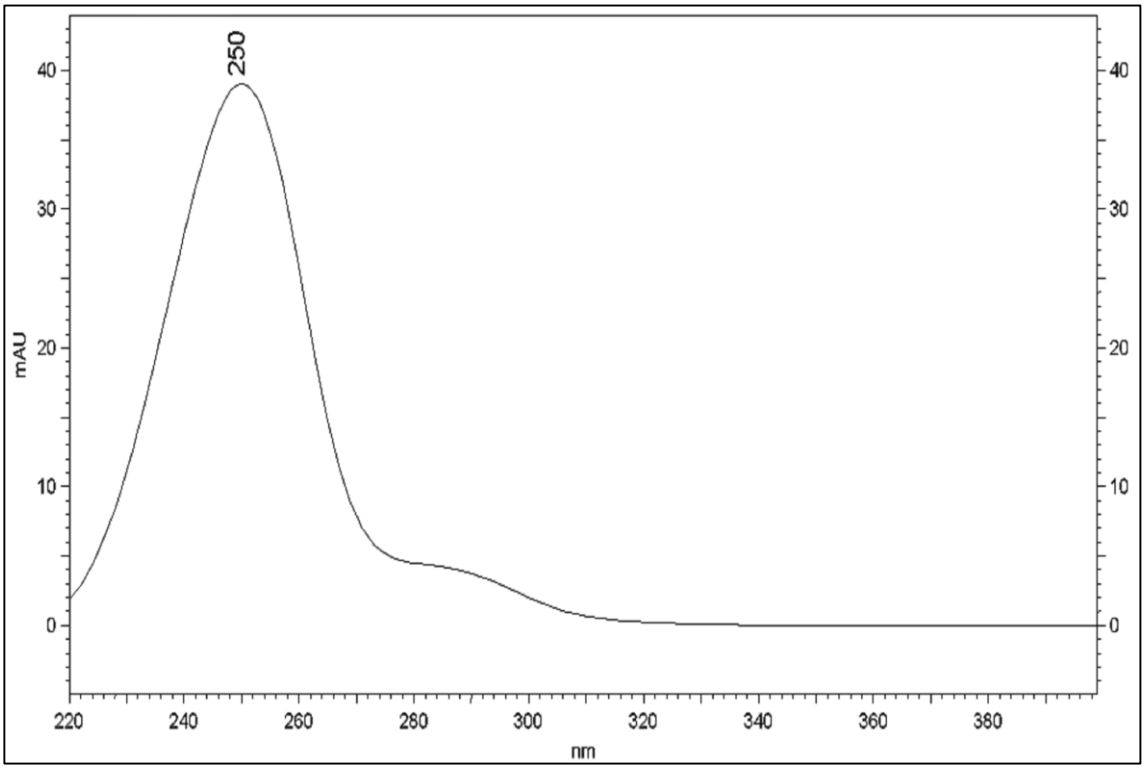


Figure 25: Spectral scanning of methcathinone by DAD (200 nm – 400nm)

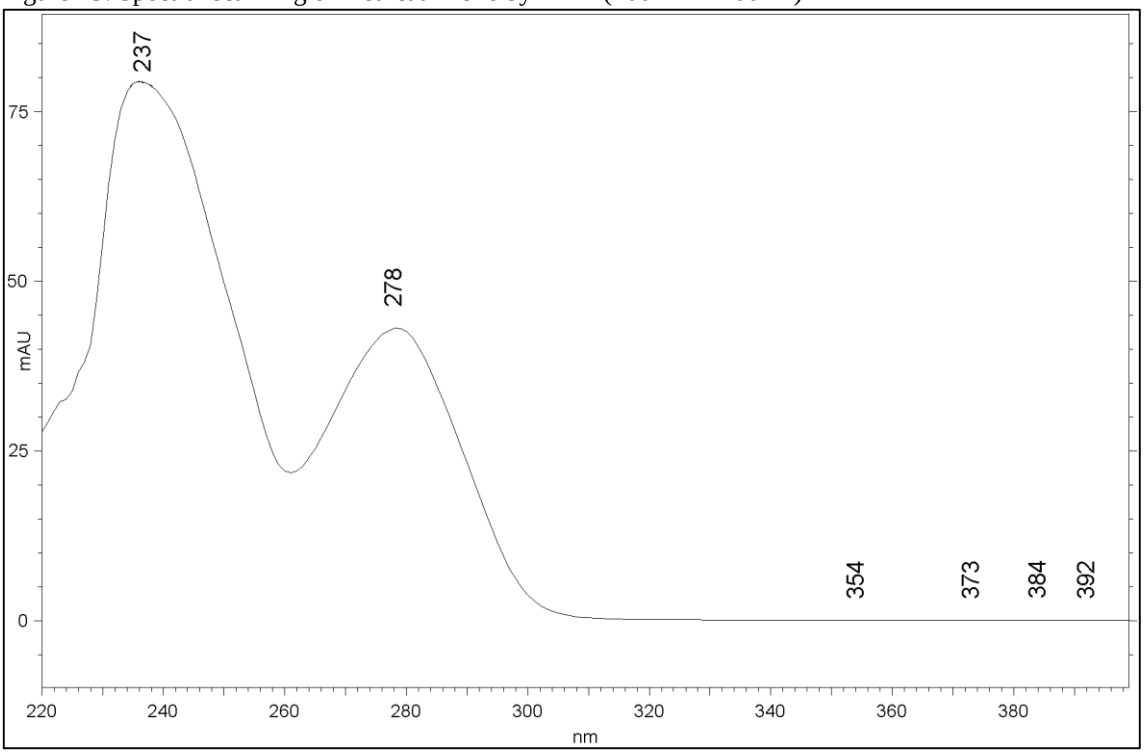
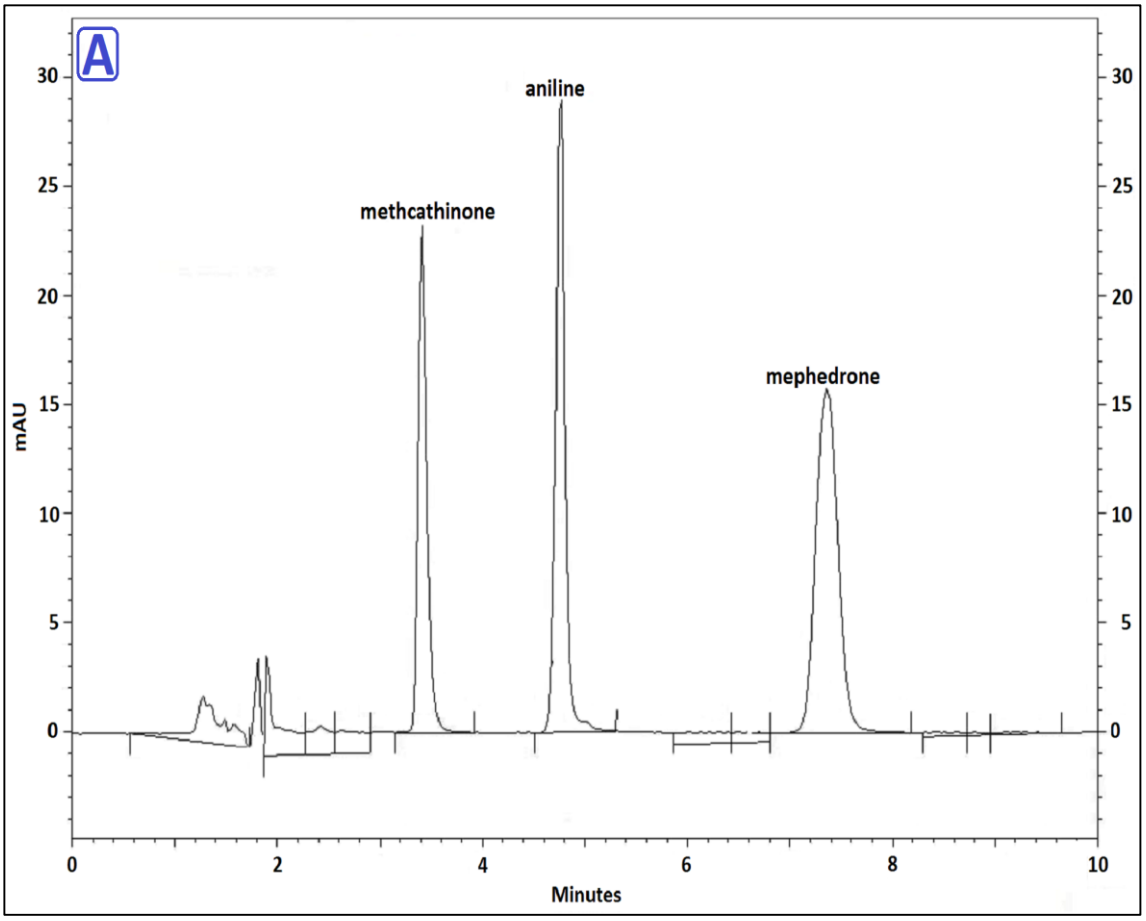


Figure 26: Spectral scanning of aniline by DAD (200 nm – 400nm)

4.1.6. Optimization of the LLE method of mephedrone and methcathinone mixture followed by analysis utilizing HPLC method (II)

The protocol for LLE simultaneous extraction of mephedrone and methcathinone from whole blood or serum matrix, as described in section 03.1.3.6. , has produced significant improvement in the percent recovery and avoidance of the emulsion formation problem by choosing the type of solvent and avoiding extreme pH values during LLE process. The efficacy of this newly developed LLE protocol was observed qualitatively through chromatograms, which showed the appearance of three distinctive separated peaks corresponding to the analytes, mephedrone and methcathinone, and the internal standard with minimal interfering peaks. The identity of each analyte peak was confirmed by its retention time and the wavelength of maximum absorbance, which were determined previously. The chromatograms showed satisfactory resolution between the analytes and the internal standard, for samples extracted from whole blood or serum matrix (Figure 27)



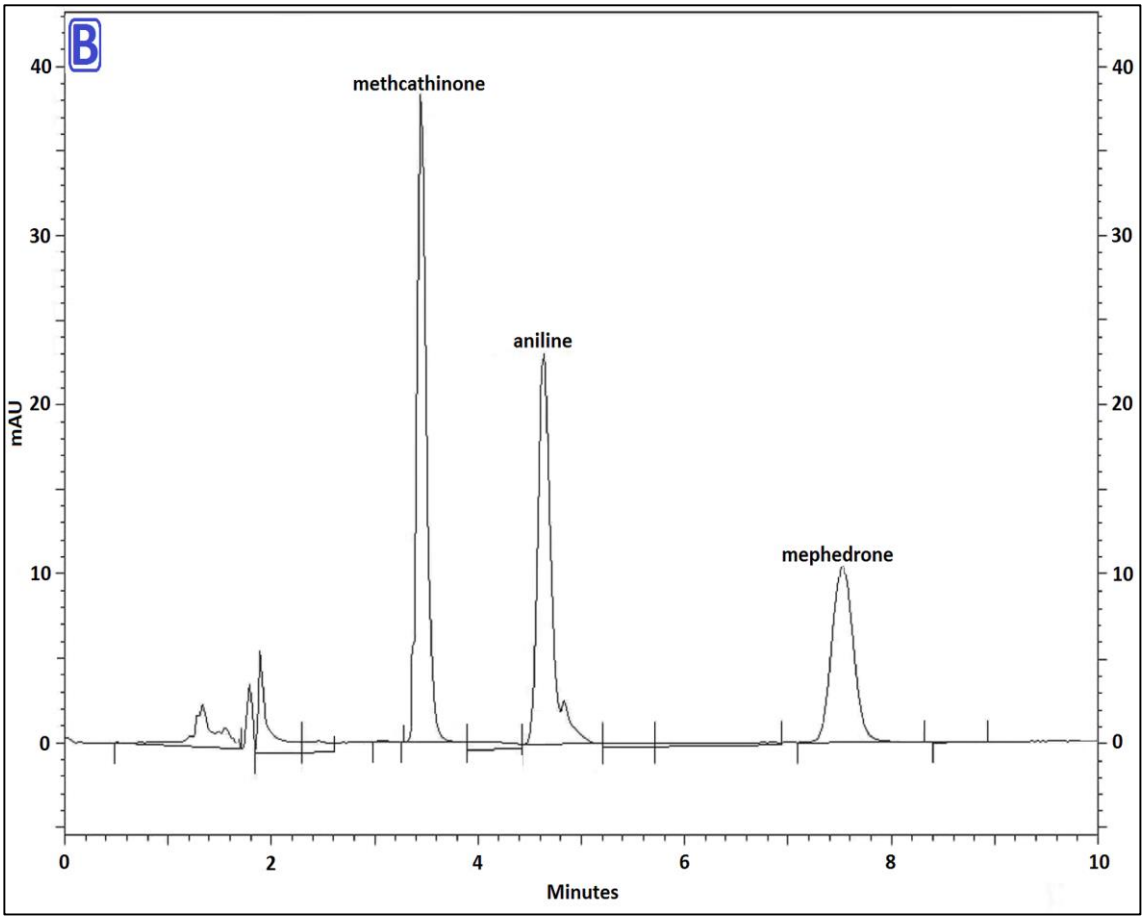


Figure 27: representative HPLC profile of the separation of 5 µg/ml mephedrone and 5µg/ml methcathinone standard in presence of the internal standard aniline after LLE from (A) blood and (B) serum samples

During the extraction procedure, emulsion formation was one of the major problems faced through the work, which is expected to negatively affect the recovery, precision and other validation parameters. Emulsion formation will cause low recovery due to low partition efficiency and volume loss (Hardy and Jones, 1997). There are many suggested procedures to prevent emulsion formation or to break any that form. Gentle shaking, avoiding the use of extreme pH values, the use of low polarity organic solvent, salting the sample, adding alcohol ...etc., are all possible precautions to take in order to minimize the effect of emulsion formation. Experimental modifications were performed to minimize the emulsion formation effect and so improve precision and accuracy including changing the pH value and type of extracting solvent. In addition to the mechanical precautions, using a less polar extraction solvent, combining the extraction solvent with alcohol and avoiding extreme high pH values may minimize emulsion formation.

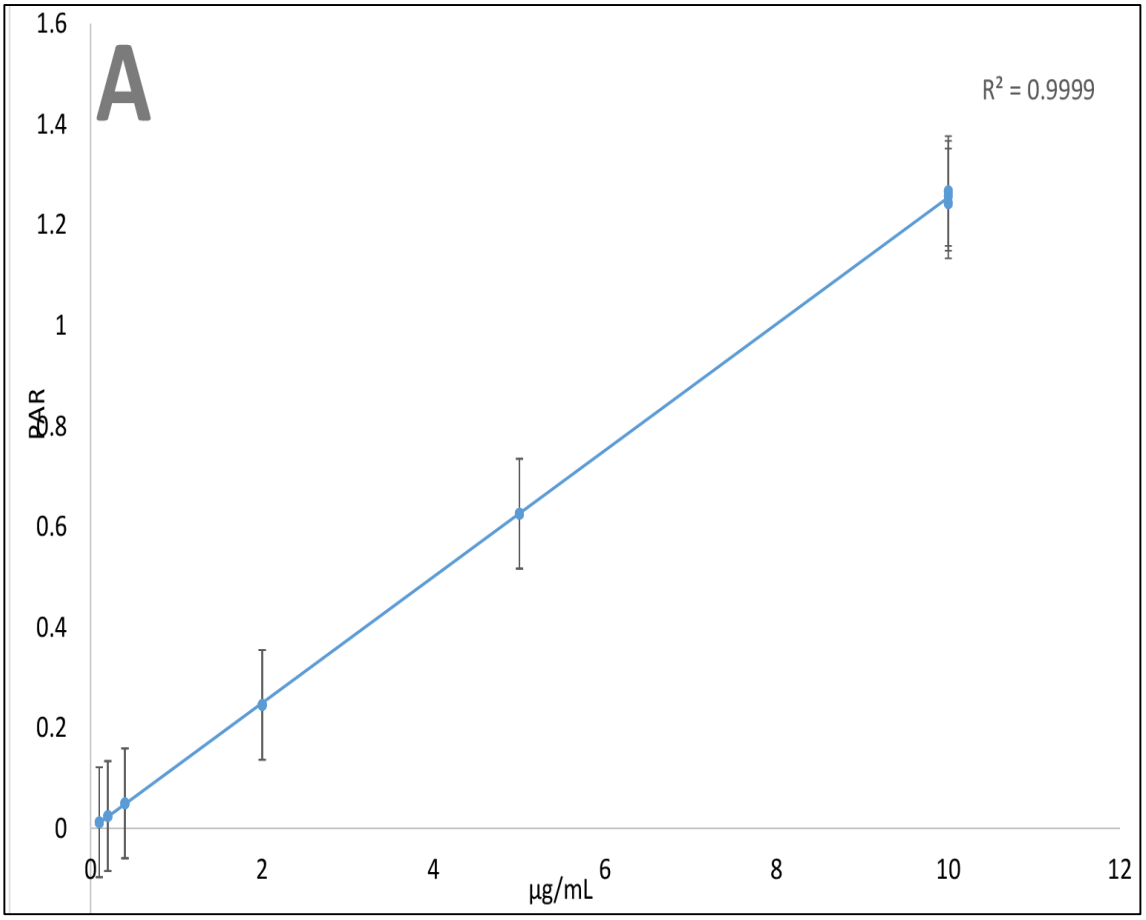
Studying the theoretical rules for the formation, stabilization and breakage of emulsions, suggested precautions maybe explained. When two immiscible liquids come in contact, the most thermodynamically stable setup is to form two separate layers to minimize the area of contact between the two liquids. To form an emulsion, energy is needed to overcome this thermodynamically stable status, which can be provided by simple mechanical forces like shaking the two liquids or using a stirrer. However, an emulsifying agent can be used. The simplest types of emulsifying agents are ions like hydroxyl ions, which may be adsorbed onto the dispersed droplets producing electrostatic repulsion forces and hence stabilization of the formed emulsion (McClements and Weiss, 2005; Tadros, 2013). The use of extreme pH may increase the chance of formed ions and charged dispersed droplets, and in turn stabilize the emulsion formed. Protein and phospholipids are other important emulsifying agents, and biological samples contain high levels of these two components (McClements and Weiss, 2005). Phospholipids are of the major component of cell membranes, but also industrially available in food technology as emulsifying agents. The high content of proteins and phospholipids in biological samples will act as emulsifying agents, and as a precaution toward minimizing their effect, protein precipitation is often performed as a pre-treatment step which will help in minimizing formation and stability of those emulsions.

Ethyl acetate is relatively polar solvent (polarity index= 4.4), and emulsion formation could be minimized by using a less polar solvent like dichloromethane (polarity index = 3.1). Dichloromethane is a volatile solvent (boiling point 39.6 C°) and immiscible with water, making it one of the possible working extraction solvents. The alcohol butanol is immiscible with water while it is miscible with dichloromethane, and has comparable polarity to ethyl acetate (polarity index= 4.0). However, the boiling point of butanol is quiet high (117.7 C°), which may decrease the volatility of the organic mixture. Different solvent mixture ratios were tested for the time required to fully evaporate under streaming N_2. The solvent mixture of dichloromethane: butanol (8:2, v: v) worked well and the mixture totally evaporated to dryness under streaming N_2 in about 5 minutes. Consequently, using the less polar extraction solvent dichloromethane, combined with the alcohol butanol, and using buffer pH 9.2 instead of pH10 worked very well and improved the LLE outcome.

4.1.7. Validation of an LLE method for mephedrone and methcathinone mixture followed by analysis utilizing HPLC method (II)

Linearity: Calibration curve of the HPLC method using standard concentrations (before extraction) was produced by plotting average peak areas against corresponding concentrations, and the plot yields R2> 0.99 for both of the drugs mephedrone and methcathinone emphasizing the linearity of the specified HPLC method over the working range of 0.2 -10 µg/ml.

The linearity of the HPLC method after extraction was assessed by plotting average peak area ratios - Drug/I.S.- against corresponding sample concentrations of mephedrone and methcathinone within the working range (0.1-10 µg/mL) for both blood and serum samples, yielding R2 > 0.99 in both matrices (Figure 28).



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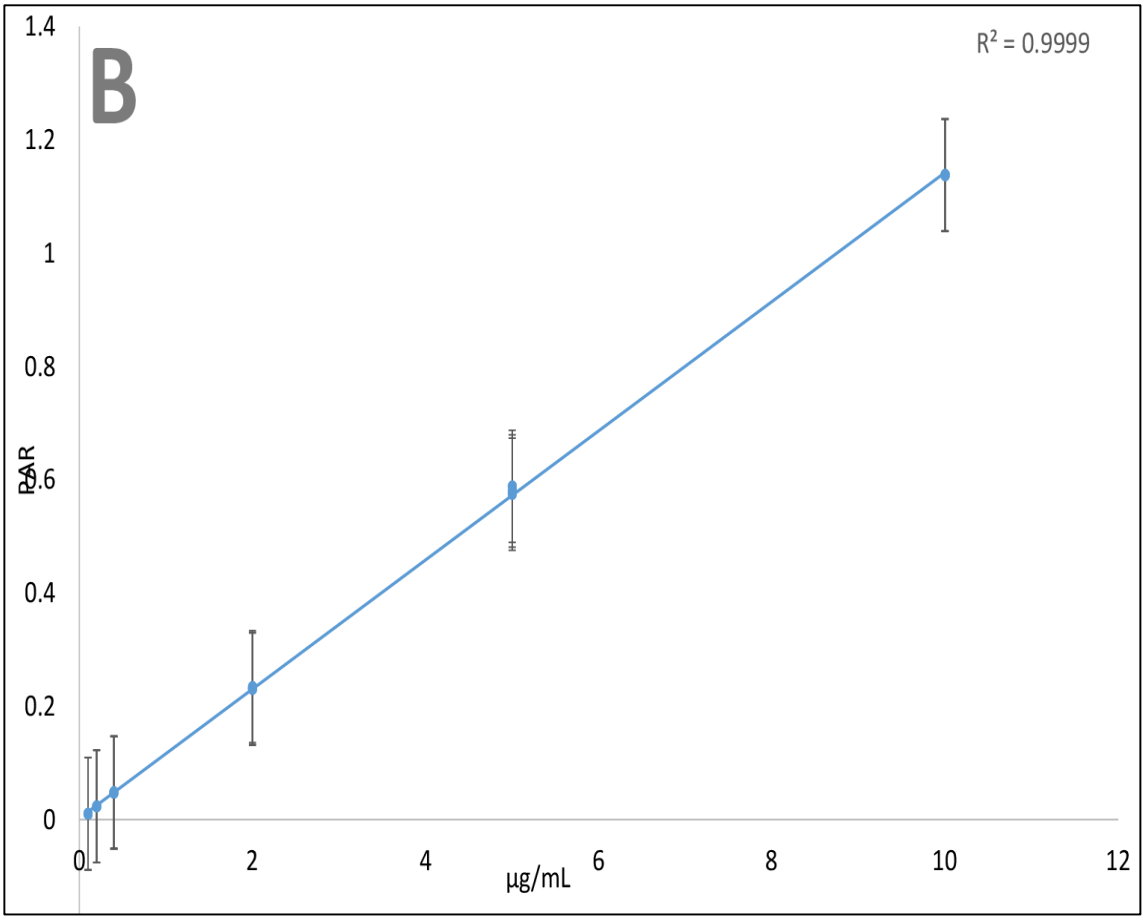
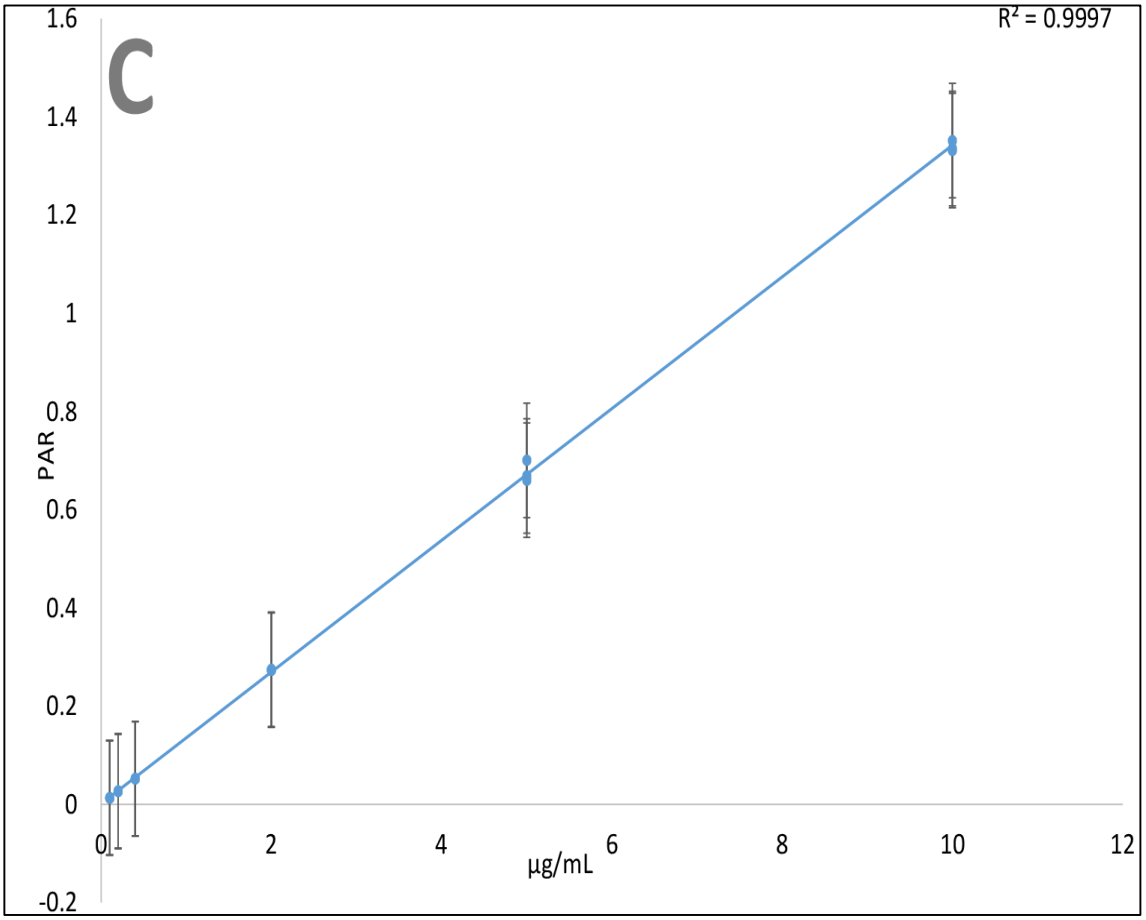

B
R² = 0.9999
PAR
µg/mL
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12


C
R² = 0.9997
PAR
µg/mL
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
2
4
6
8
10
12

Continued

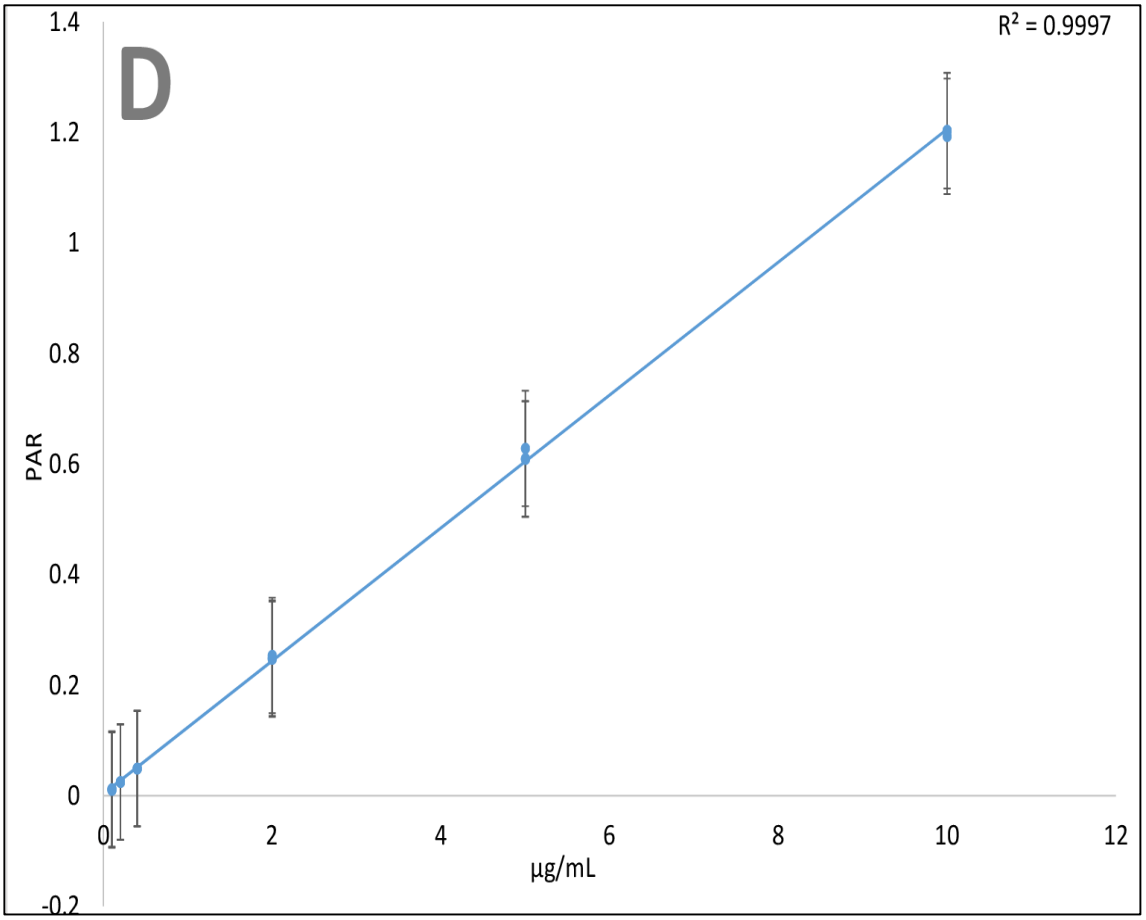


Figure 28: calibration curves of mephedrone and methcathinone standards over the specified ranges (0.1-10 µg/mL) applying developed HPLC method (II) after LLE from blood and serum matrix mephedrone from blood (A) and serum (B), methcathinone from blood (C) and serum (D)

Precision: considering the repeatability (intraday precision), the method was assessed within the same day and showed to be repeatable, for both mephedrone and methcathinone, with LLE extraction performed on blood or serum samples reflected by RSD% values (n=5). The RSD% values were nearly similar ranging between 3.01 - 3.98%. Considering Intermediate precision (inter-day precision) the method was assessed over different days and showed to be intermediately precise, for both mephedrone and methcathinone, with LLE extraction performed on blood or serum matrices, reflected by RSD% values (n=3). The RSD% values were nearly similar ranging 4.16 - 6.73%, and no matrix effect was noticed for both drugs.

Accuracy was assessed by calculating recovery percentages of the tested drugs at different days, drug controls were prepared to allow the calculation of recovery percentages.

Considering specificity, chromatograms showed good resolution between peaks, and one pure peak for each drug and the internal standards was recorded. The purity of the peaks utilizing HPLC-DAD was confirmed by computing the ratio of signals at every point across the peak at different wavelengths. A constant ratio, which will produce a rectangular form, indicates a pure peak- i.e. no interference, and so specificity to the corresponding analyte. Any distortion to the rectangular form indicates differential absorbance indicating the presence of impurities (Papadoyannis and Gika, 2005; Snyder et al., 2009). Theoretically, any two wavelengths in the spectrum can be used to compute the ratio of absorbance. However, most of the analytes have maximum absorbance at 258 nm (Ardrey, 2003), and practically the measured maximum absorbance of mephedrone and methcathinone were determined to be 262 and 250 nm respectively. For that, the 240 and 280 nm wavelengths, which cover that range of the determined maximum absorbance of the major analytes, were used to compute peak purity by calculating the ratio. Mephedrone, methcathinone and the internal standard ratiograms produced the typical rectangular form which reflect the purity of the produced peaks and hence specificity of the developed HPLC method (Figure 29).

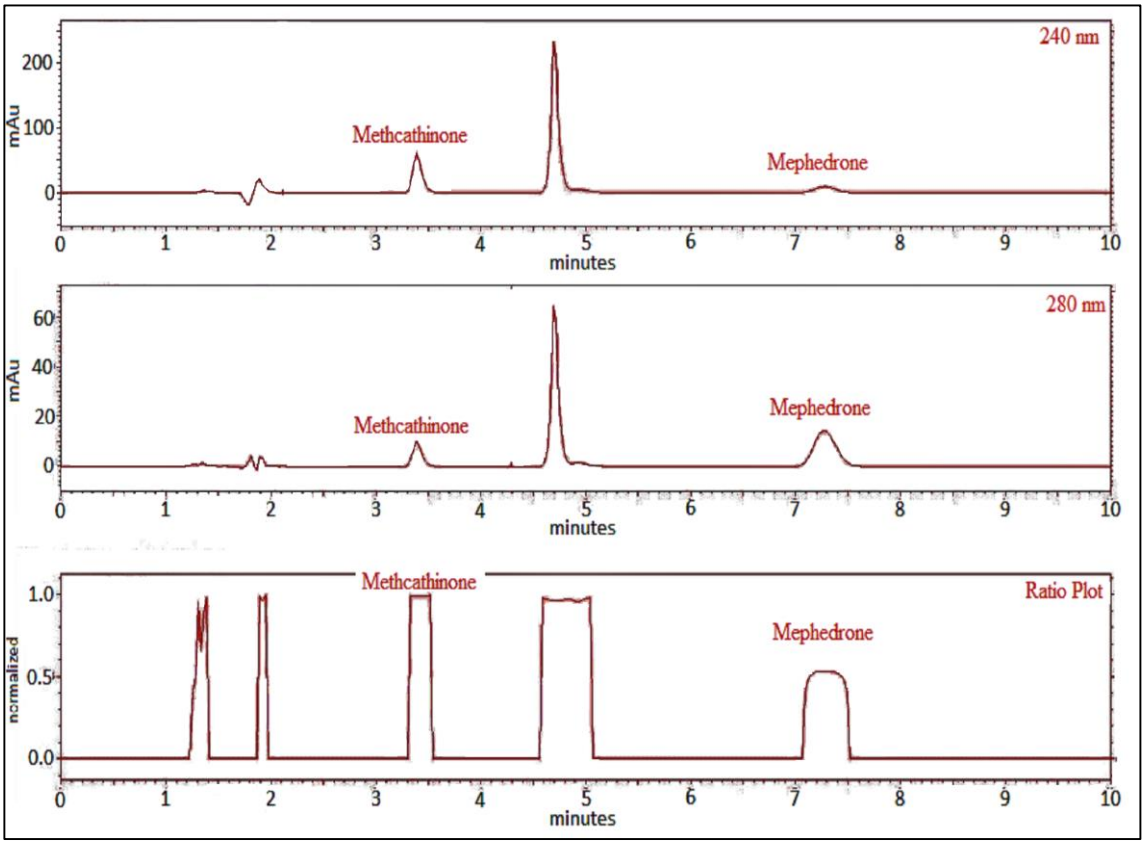


Figure 29: Peak ratios at wavelengths 240/280 nm for the assessment of peak purity

Limit of detection and Limit of quantitation were calculated mathematically applying the previously mentioned equations in section 3.1.3.3. Peak areas were applied in the above mentioned equation instead of peak heights. The minimum tested sample concentration with acceptable RSD was 0.1 µg/mL for both drugs. Peak areas of the minimum concentration with accepted RSD% value were nearly comparable in both matrices ranging between 16289 - 19699 and between 19309 - 23580 for mephedrone and methcathinone, respectively. Average background noise was calculated to 350, and peak areas were applied instead of the peak heights. LOD ranged between 0.010 and 0.013 µg/mL and LOQ ranged between 0.032 and 0.043 µg/mL for both drugs extracted from both matrices. Summary of the validation parameters are presented in Table 13.

Table 13: Summery of the validation parameters for LLE of mephedrone and methcathinone mixture followed by analysis utilizing HPLC -DAD

Drug	matrix	Linearity	Repeatability	Intermediate precision	recovery%	LOD[1] µg/ml	LOQ[2] µg/ml
mephedrone	Blood	>0.99	3.01%	6.73%	84-109%	0.011	0.036
	Serum	>0.99	4.30%	5.03%	80-108%	0.013	0.043
methcathinone	Blood	>0.99	3.98%	6.33%	83-110%	0.010	0.032
	Serum	>0.99	3.69%	4.16%	81-106%	0.011	0.036

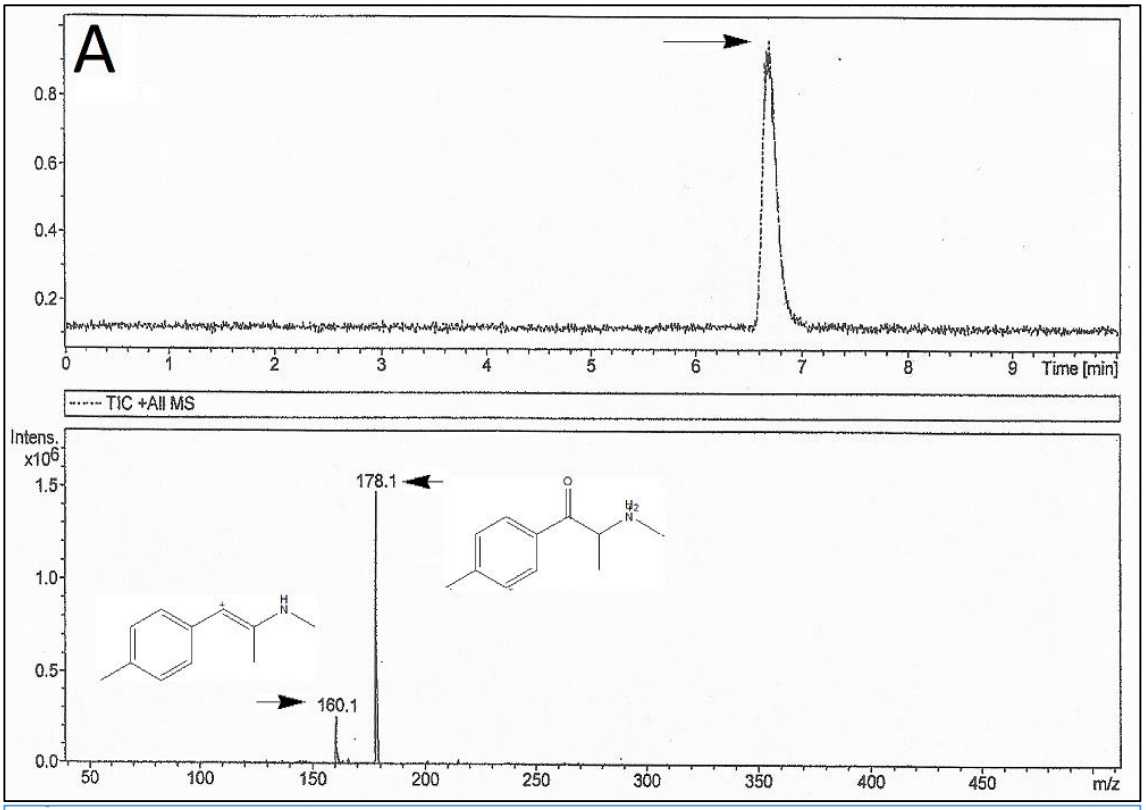
4.2. *In vitro* metabolism studies of selected NPS using pig liver microsomes followed by analysis utilizing LC-MS

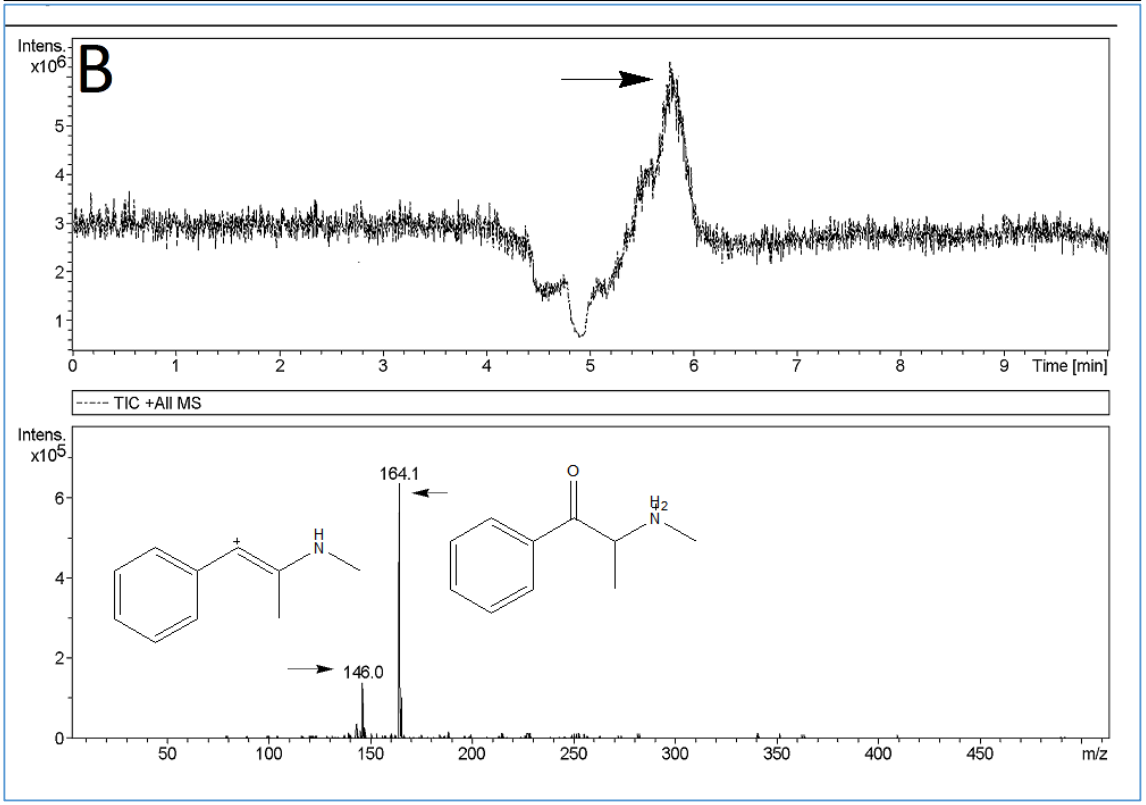
Mephedrone is structurally related to cathinones (MW=177), relatively polar compounds with a peripheral tolyl moiety and beta ketoamine backbone. Standards of mephedrone were injected onto the LC-MS system and eluted at 6.7 min. The mass spectrum of the detected peak showed abundant MH+ ions at m/z= 178 that is an evidence of the drug molecular weight. Another m/z value of 160 was identified that is produced from neutral loss of water.

For methcathinone (MW=163), it eluted at 5.8 min. The mass spectrum of the detected peak showed a similar pattern to the one observed for mephedrone. Two m/z values were identified: the abundant MH+ ions at m/z= 164 which is an evidence of the drug molecular weight and the m/z value of 146, the product of neutral loss of water.

For MXE (MW=247), the drug eluted at 7.6 min. The mass spectrum of the detected peak showed three identifiable m/z values of the molecular ion and two fragments of m/z values 175 and 203 (Figure 30).

After incubation of the drugs with the metabolic system, new peaks appearing were investigated, and proposed structures for the proposed detected metabolites were determined from the fragments present in the respective mass spectra and interpreted in correlation to those of the parent drug. Negative controls were used to verify the absence of these metabolites in the absence of these drugs. No new identifiable peaks were detected when injecting the sample produced from the incubation of the selected drugs with S9 fraction. It was possible to identify some newly appearing peaks for the drugs mephedrone and methoxetamine when injecting the samples produced from the incubation of these drugs with microsomes, while no new identifiable peaks where detected when injecting the samples produced from the incubation of methcathinone and MDAI with microsomes.


A
Time [min]
TIC +All MS
Intens.
x10^6
178.1
160.1
m/z


B
Intens.
x10^6
Time [min]
TIC +All MS
Intens.
x10^5
164.1
146.0
m/z

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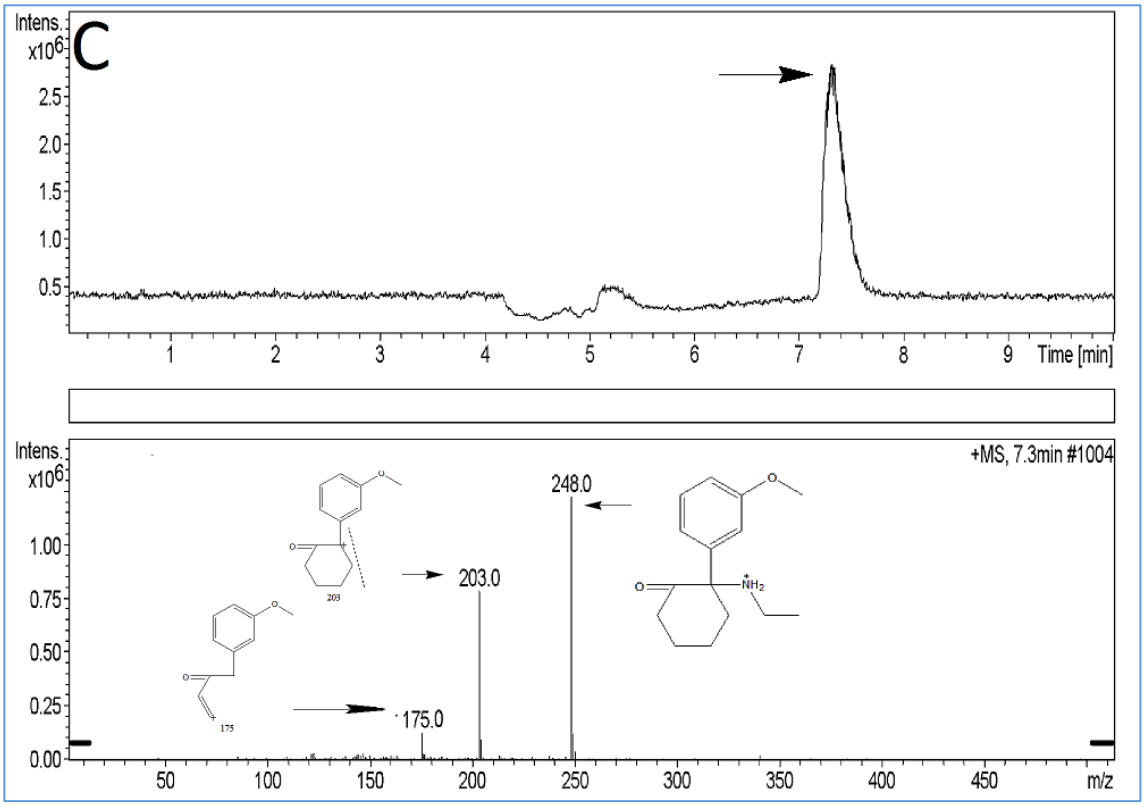


Figure 30: Representative chromatogram and mass spectrum for each of the selected drugs produced utilizing LC-MS., with the proposed structure of detected fragments
(A): mephedrone, (B): methcathinone and (C): methoxetamine

4.2.1. Mephedrone

Mephedrone was incubated with in-house prepared pig liver microsomes under the specified conditions and after termination of the metabolic process, samples were centrifuged, filtered and injected onto the LC-MS system. The possible metabolic pathway that mephedrone will go through can be proposed depending on published data about structurally related drugs and other *in vivo* or *in vitro* studies about the metabolism of mephedrone. Meyer et al. utilised GC-MS for *in vivo* studies and detection of mephedrone metabolites in human and rat urine. They concluded that mephedrone may be metabolised through phase 1 enzymatic system through demethylation, reduction or oxidization. Demethylation involves the removal of the N-methyl functional group producing nor-mephedrone (IUPAC: 2-Amino-1-p-tolyl-propan-1-one, MW=163) which will go further into reduction of the ketone moiety producing the corresponding alcohol, nor-dihydro mephedrone (IUPAC: 2-Amino-1-p-tolyl-propan-1-ol, MW: 165). Also, hydroxytolyl-mephedrone (IUPAC: 1-(4-Hydroxymethyl-phenyl)-2-methylamino-propan-1-one, MW=193) and nor-hydroxytolyl

mephedrone (2-Amino-1-(4-hydroxymethyl-phenyl)-propan-1-one, MW=179) in addition to 4-carboxy-dihydro mephedrone (IUPAC: 4-(2-Amino-1-hydroxy-propyl)-benzoic acid, MW=195) were detected at their study as a product of an overlapping metabolic pathway of oxidation, reduction and N-demethylation. It was concluded through their study also that phase II metabolic pathways are involved in the metabolism of mephedrone through sulphonation or glucuronidation of the hydroxytolyl- mephedrone and its demethylated form (Meyer et al., 2010).

Accordingly, a possible metabolic pathway can be proposed for mephedrone (Figure 31), which is useful for the prediction and detection of metabolites by tracing specific m/z values and extraction the chromatograms for specific ion mass. As noticed, three major metabolic reactions are involved that may overlap, i.e. the drug may go through more than one metabolic reaction step producing different metabolites. Chromatograms were traced and extracted for the selected masses that correspond to the molecular ions (MH+) of each of the suggested metabolites, and further the detected mass spectra peaks were analyzed for the fragmentation pattern in relation to the one observed for the drug mephedrone. The suggested metabolites either contain a β-ketone moiety which may produce (MH+ - 18) ion due to neutral loss water or the reduced form of the corresponding alcohol which may either produce (MH+ - 18) or (MH+ - 17) due to neutral loss of water or hydroxyl, respectively (Table 14).

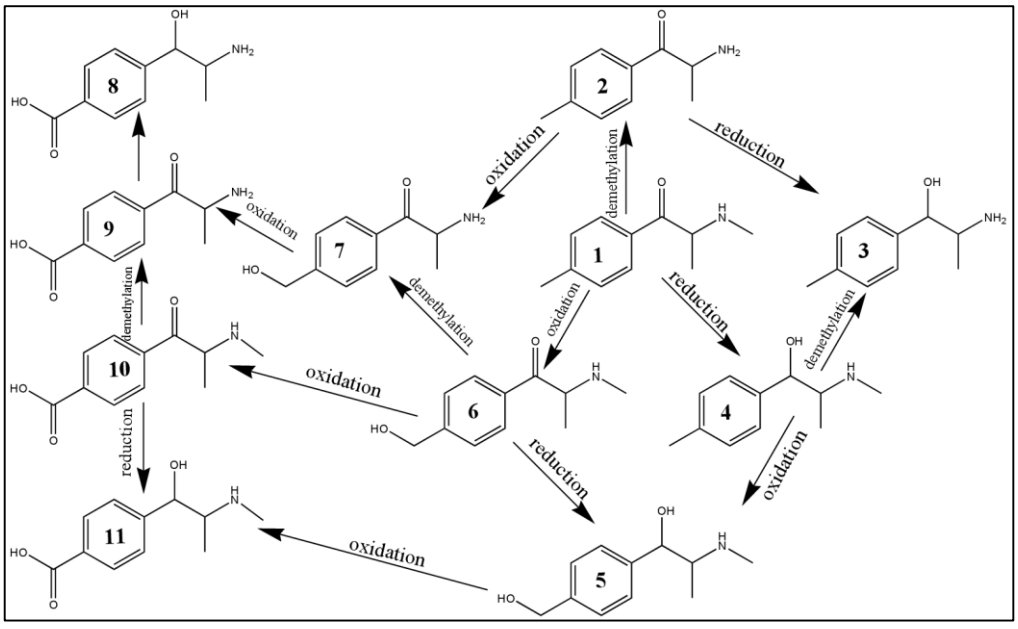


Figure 31: Suggested phase I metabolic pathway of mephedrone adapted from published data for the metabolism of mephedrone and similar drugs.

Table 14: Suggested metabolites produced from phase I metabolism of mephedrone after incubation with liver microsomes and analysis utilizing LC-MS/IOT and the monitored ion masses

Number[1]	Name	MW[2]	Extracted MH^+	$-H_2O$[3]	-OH[4]
1	**mephedrone**	**177**	**178**	**160**	**----**
2	**nor-mephedrone**	**163**	**164**	**146**	**---**
3	**nor-dihydro-mephedrone**	**165**	**166**	**148**	**149**
4	**dihydro mephedrone**	**179**	**180**	**162**	**163**
5	**4-hydroxytolyl-dihydro-mephedrone**	**195**	**196**	**178**	**179**
6	**4-hydroxytolyl-mephedrone**	**193**	**194**	**176**	**---**
7	**4-hydroxytolyl-nor-mephedrone**	**179**	**180**	**162**	**---**
8	**4-carboxy-nor-diydro-mephedrone**	**195**	**196**	**178**	**179**
9	**4-carboxy-nor-mephedrone**	**193**	**194**	**176**	**---**
10	**4-carboxy-mephedrone**	**207**	**208**	**190**	**---**
11	**4-carboxy-diydro-mephedrone**	**209**	**210**	**192**	**193**

1: molecule number in reference to Figure 31 2: molecular weight
3: neutral loss of water 4: loss of hydroxyl

Total ion chromatogram(TIC) was extracted for the molecular ion of mephedrone and of each of the suggested metabolites. Extracted ion chromatograms (EIC) produced three peaks where one of the mass spectra is that of the parent drug mephedrone while the other two mass spectra were suggestive to be one of the metabolites of mephedrone. The detected peaks that were suggestive of the metabolites of mephedrone had a spectrum with abundant MH+=194 and MH+=166 (Figure 32).

The detected peak with MH+=178 appeared at 6.6 minutes and has mass spectrum with abundant MH+=178 and secondary fragment of m/z= 160 (MH+=178-18) which confirm its identity of as the parent drug mephedrone in compare to the previously determined retention time and fragmentation pattern of mephedrone (see Figure 30)

The detected peak with MH+=194 appeared at 5.4 minute and has mass spectrum with abundant MH+=194 and secondary fragment of m/z= 176 (MH+=194-18). The detected peak with MH+=166 appeared at 6.3 minute and has mass spectrum with abundant MH+=166. Following the suggested metabolic pathway, it is proposed that the produced metabolite with MH+=194 is either hydroxytolyl mephedrone or 4-carboxy-nor-mephedrone while the other with MH+ =166 is nor-dihydro-mephedrone (Figure 33).

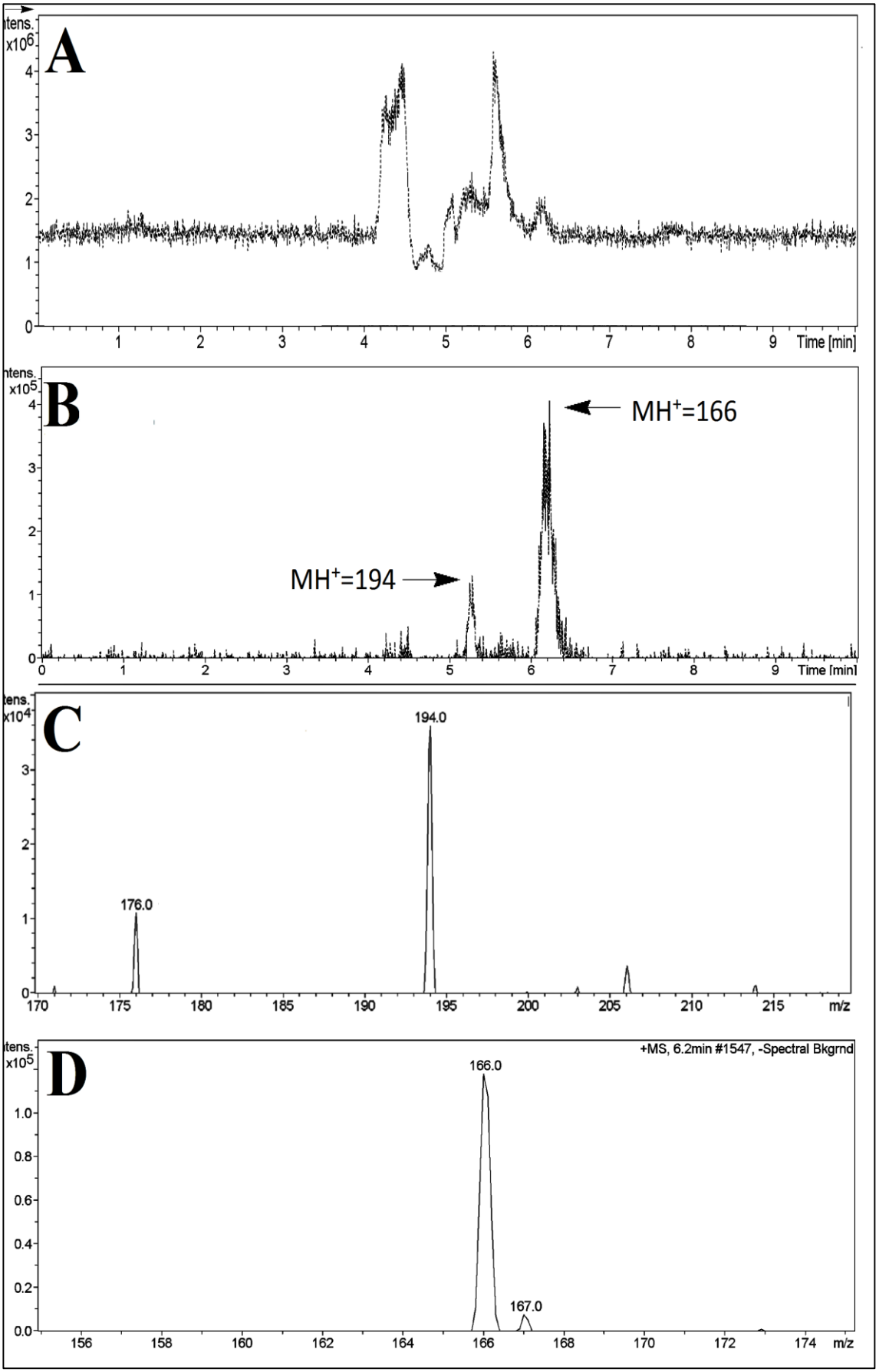


Figure 32: Representative chromatograms and spectra of the detected peaks suggestive of mephedrone metabolites after incubation with pig liver microsomes
A: TIC, B: EIC and C: mass spectrum of detected peak with MH+=194 and D: mass spectrum of detected peak with MH+= 166.

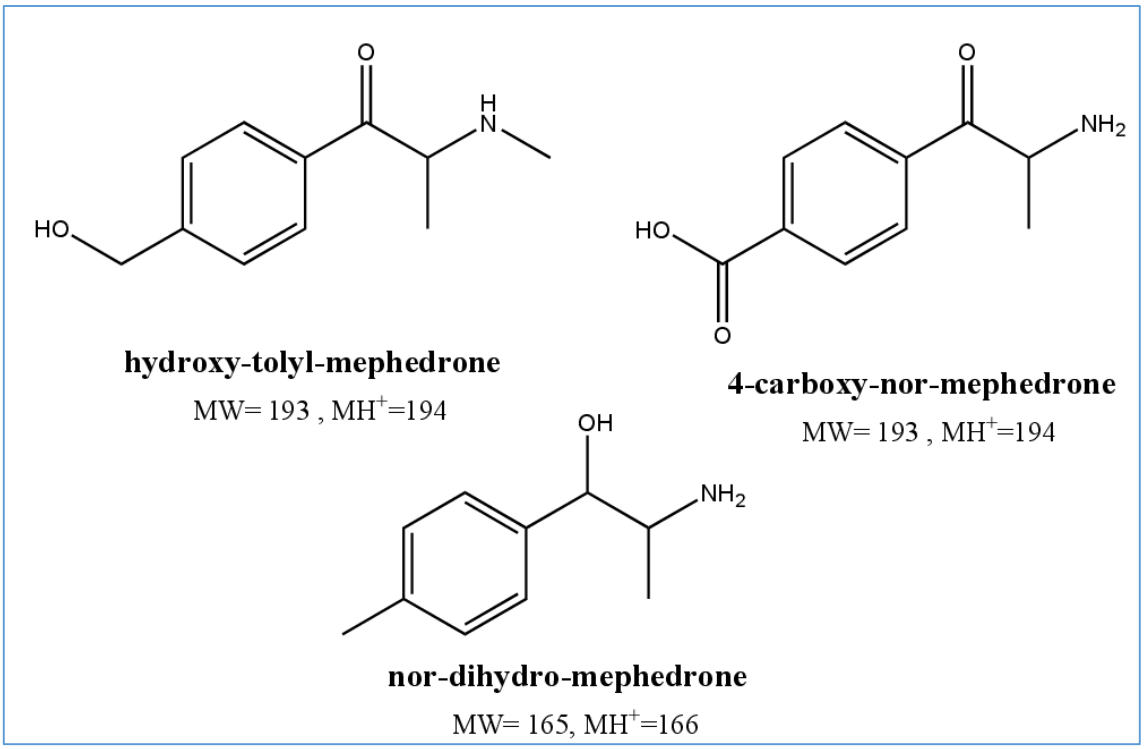


Figure 33: proposed metabolites of the identified peaks for the drug mephedrone utilizing LC-MS after incubation with in-house prepared microsomes

Samples were analysed in both selected ion monitoring (SIM) and selected reaction monitoring (SRM) modes. The selected ions were those of the molecular ion of mephedrone (MH+=178) and of the suggested metabolites (MH+= 194 and MH+=166). Analysis of the samples in SIM mode with selection of ion mass=178 produced a chromatogram with one representative peak at 6.7 min, where its mass spectrum contains abundant MH+=178. Analysis of the samples in SRM mode with selection of ion mass=178 produced a chromatogram with one representative peak at 6.7 min, where its mass spectrum contains the fragment of *m/z*=160 which is due to neutral loss of water in similar way to the one previously observed in MS analysis of mephedrone. Retention time, abundant molecular ion and typical fragmentation pattern were confirmative to the identity of the drug mephedrone (Figure 34).

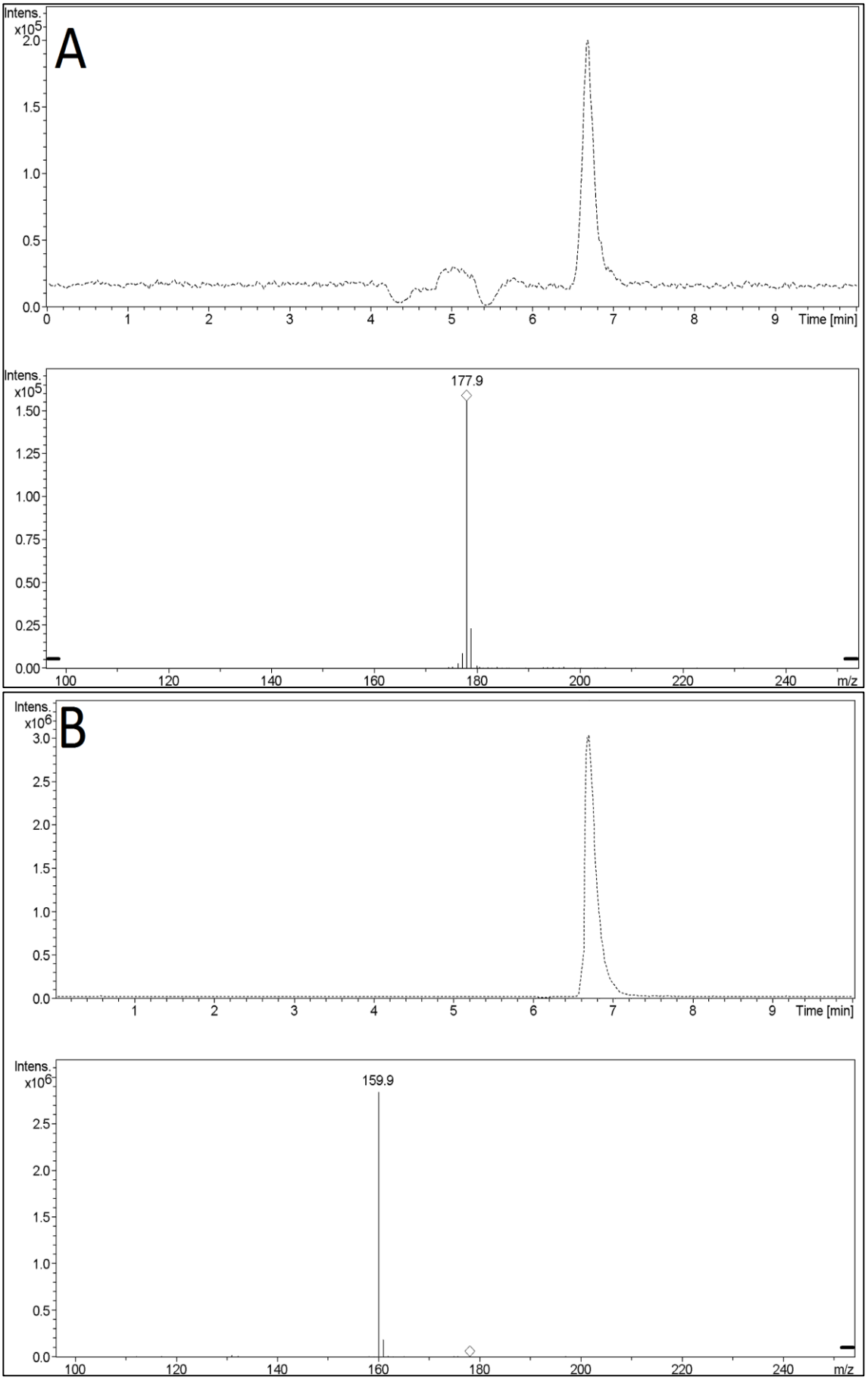
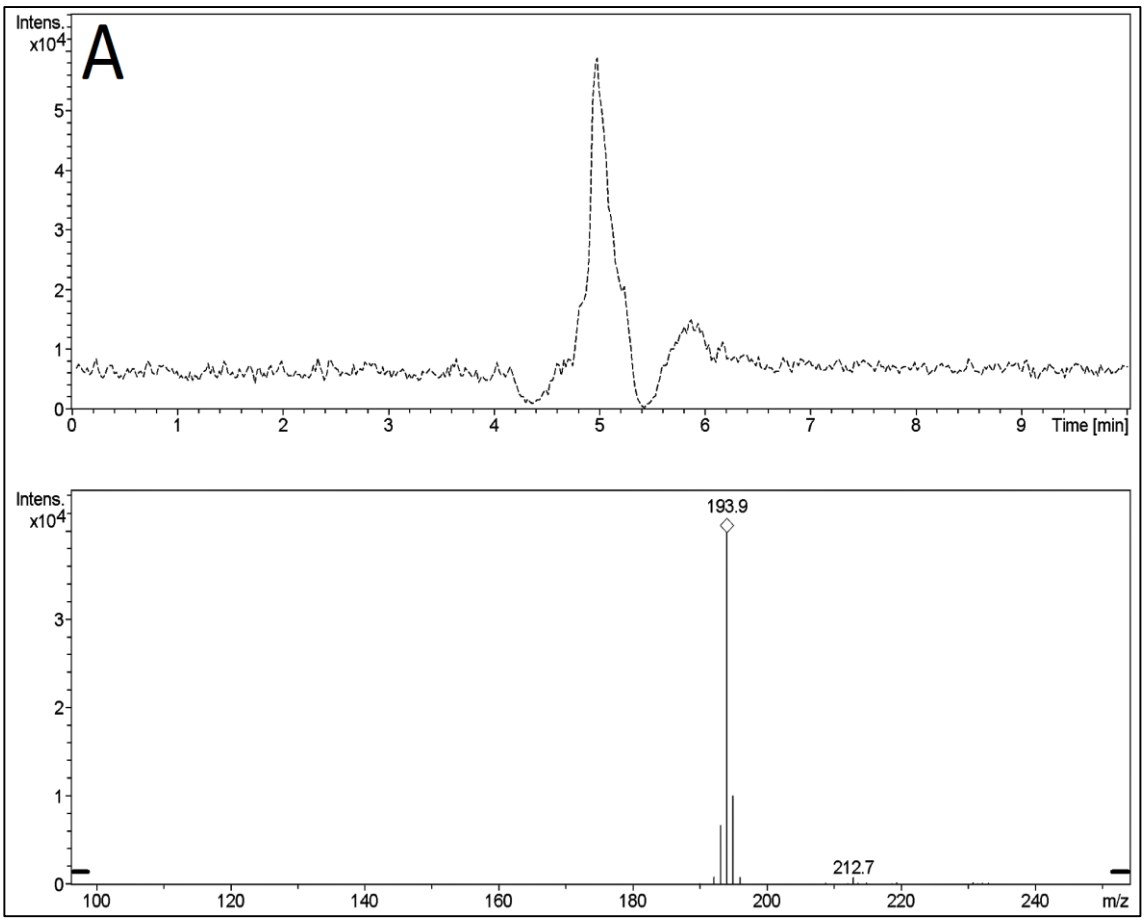


Figure 34: Representative chromatograms and spectra produced after Tandem MS. of ion mass=178 detected after incubation of mephedrone with pig liver microsomes
A: SIM mode and B: SRM mode.

Similarly, analysis of the samples in SIM mode with selection of ion mass=194 produced a chromatogram with one peak at 5.0 min, where its mass spectrum contains abundant MH+=194. Analysis of the samples in SRM mode with selection of ion mass=194 produced a chromatogram with one peak at 5.0 min. The mass spectrum of the detected peak showed one fragment with m/z= 176 which is due to neutral loss of water in similar way to the one previously observed in MS analysis of mephedrone and the detected peak at retention time =5.0. Two other fragments were detected: m/z=158, 146 and 133 (Figure 35). The fragmentation pattern of the suggested metabolites with MH+= 194 could be expected and compared to the detected fragments produced from MS/MS analysis (Figure 36)



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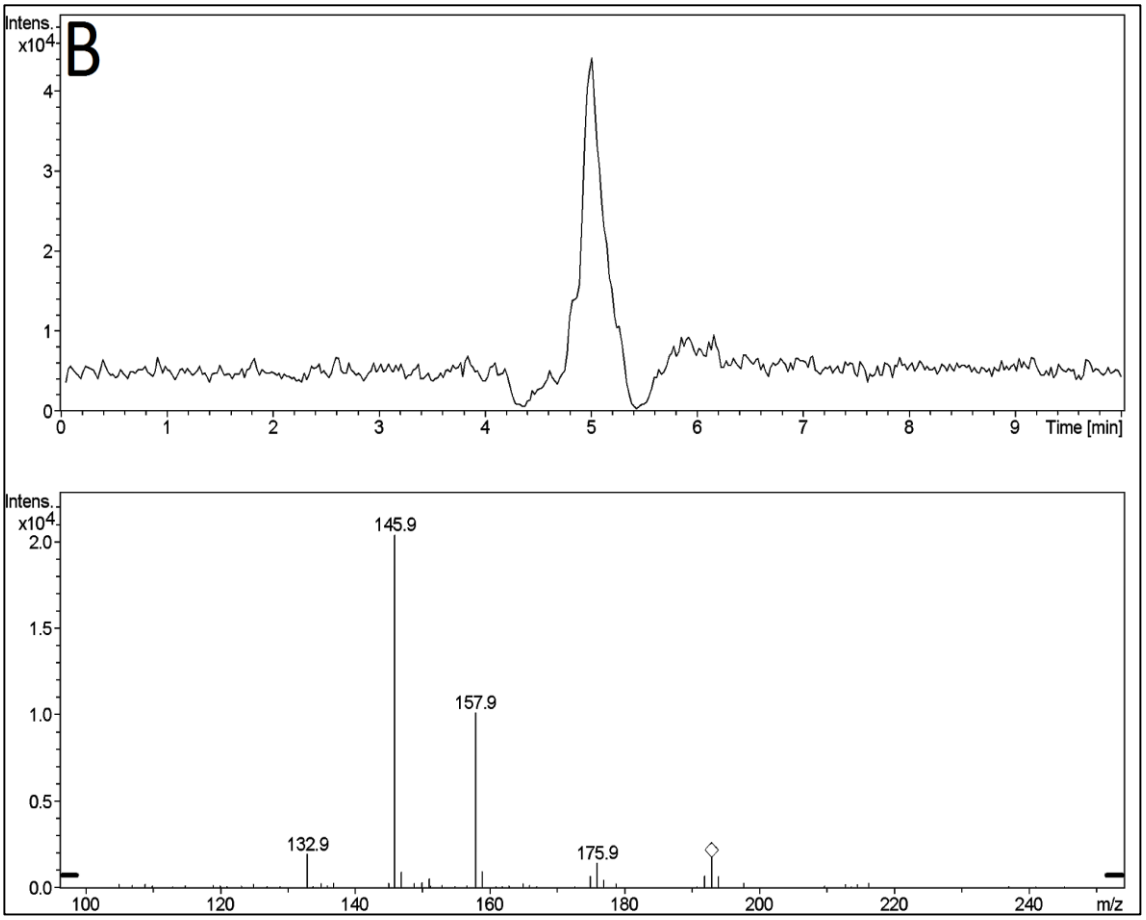
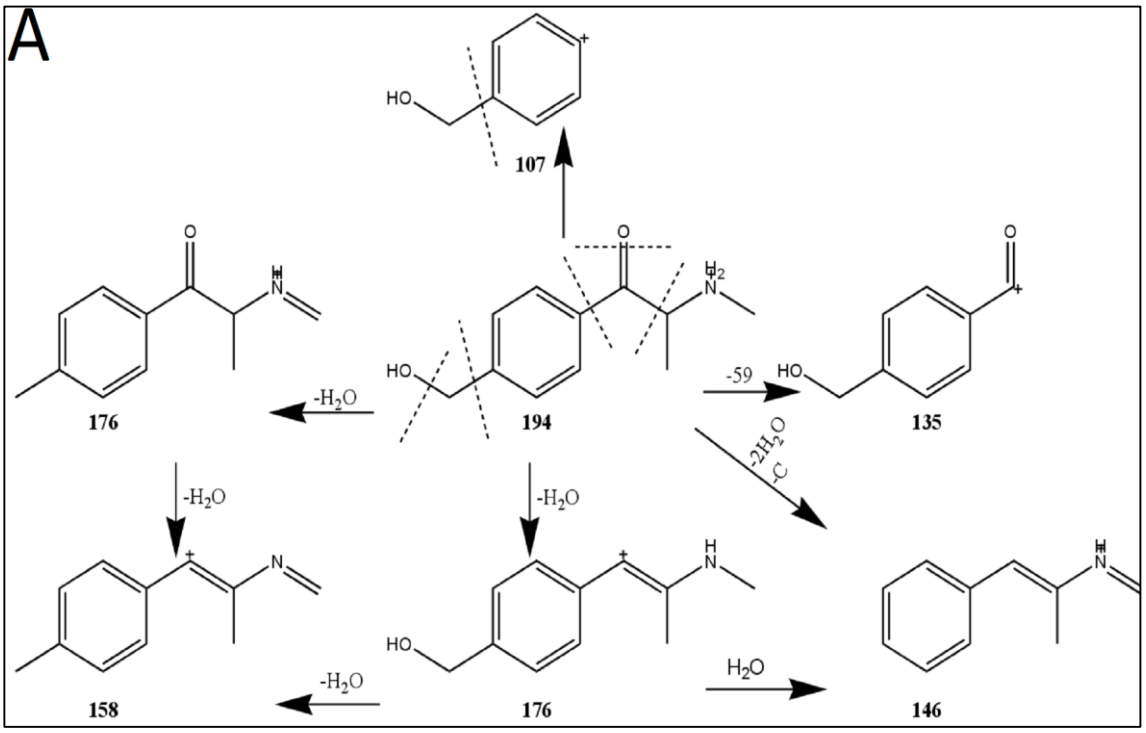


Figure 35: Representative chromatograms and spectra produced after Tandem MS. of ion mass=194 detected after incubation of mephedrone with pig liver microsomes
A: SIM mode and B: SRM mode.



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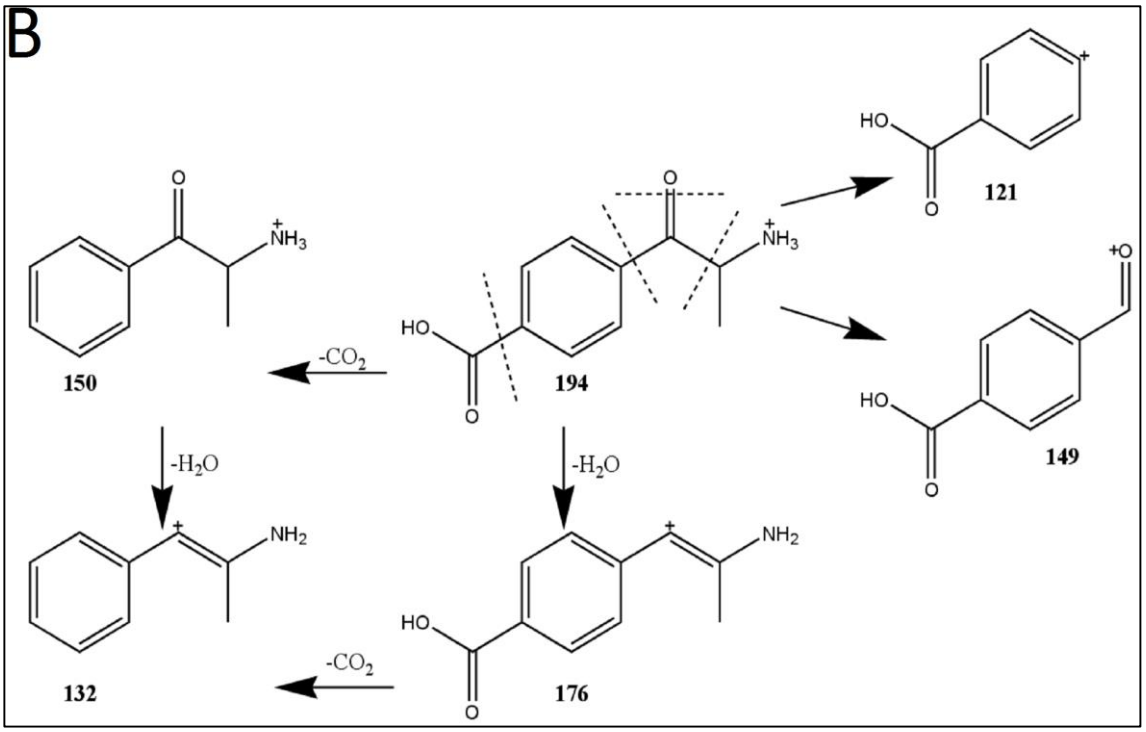


Figure 36: expected fragmentation pattern for the suggested metabolites of the peak detected of MH^+=194 after incubation of mephedrone with pig liver microsomes.
A: hydroxytolyl-mephedrone B: 4-carboxy-nor-mephedrone

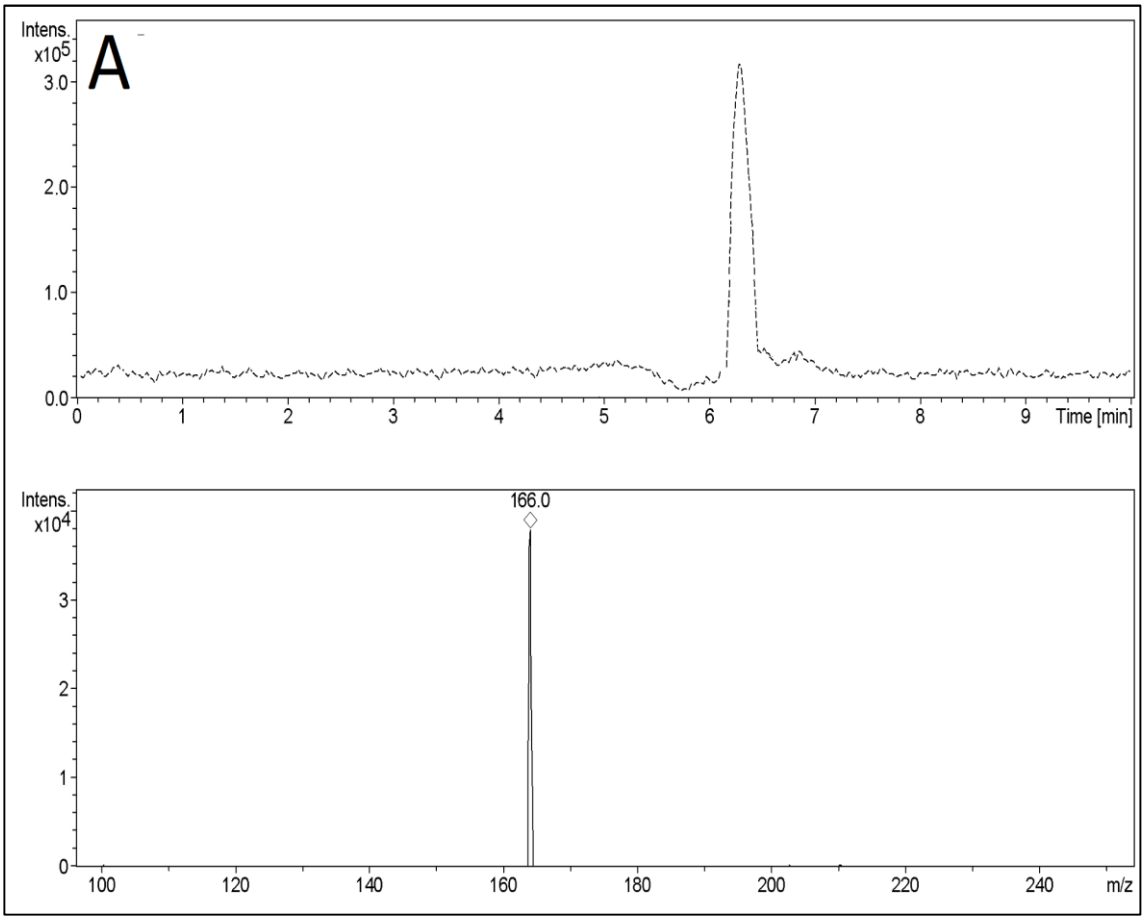
When the detected fragments in the relevant mass spectra after tandem mass analysis are compared to expected fragment of both suggested metabolites, it is more suggestive that the produced metabolite is hydroxytolyl mephedrone rather than 4-carboxy-nor-mephedrone. The proposed fragmentation pattern of hydroxyl-tolyl-mephedrone ion after tandem MS shows that the fragments are a product of one or more fragmentation steps of the parent ion, where the largest fragment of *m/z*=146 is due net loss of one carbon atom and two water molecules. The next fragment of *m/z*= 158 is due net loss two water molecules and fragment of *m/z*= 176 is due to neutral loss of water.

When performing analysis of the samples in SIM mode with selection of ion mass=166 produced a chromatogram with one representative peak at 6.3.0 min, where its mass spectrum contains abundant MH+=166. Analysis of the samples in SRM mode with selection of ion mass=166 produced a chromatogram with one peak at 6.3 min.

The mass spectrum of the detected peak showed one major fragment of *m/z*= 120 and another secondary fragment of *m/z*=149. The detected fragment of *m/z* = 149 is comparable to the fragments produced earlier for mephedrone and the suggested metabolite hydroxytolyl mephedrone. However,

the suggested metabolite of *m/z*= 166 is proposed to be produced by more than one metabolic steps where one of them is reduction of the ketone moiety to the corresponding alcohol – nor-dihydro-mephedrone. In the latter, the major fragment of *m/z*=149 is a product of neutral loss of hydroxyl group compared to neutral loss of water in MS/MS analysis of mephedrone and hydroxytolyl-mephedrone (Figure 37).

The proposed fragmentation pattern through detected shows two main fragments, the largest one due to α-cleavage with *m/z*=120 while the other with *m/z*=149 is due to neutral loss of water, though fragmentation of the peripheral amine functional group is also proposed to produce fragment of *m/z*=149 (Figure 38).



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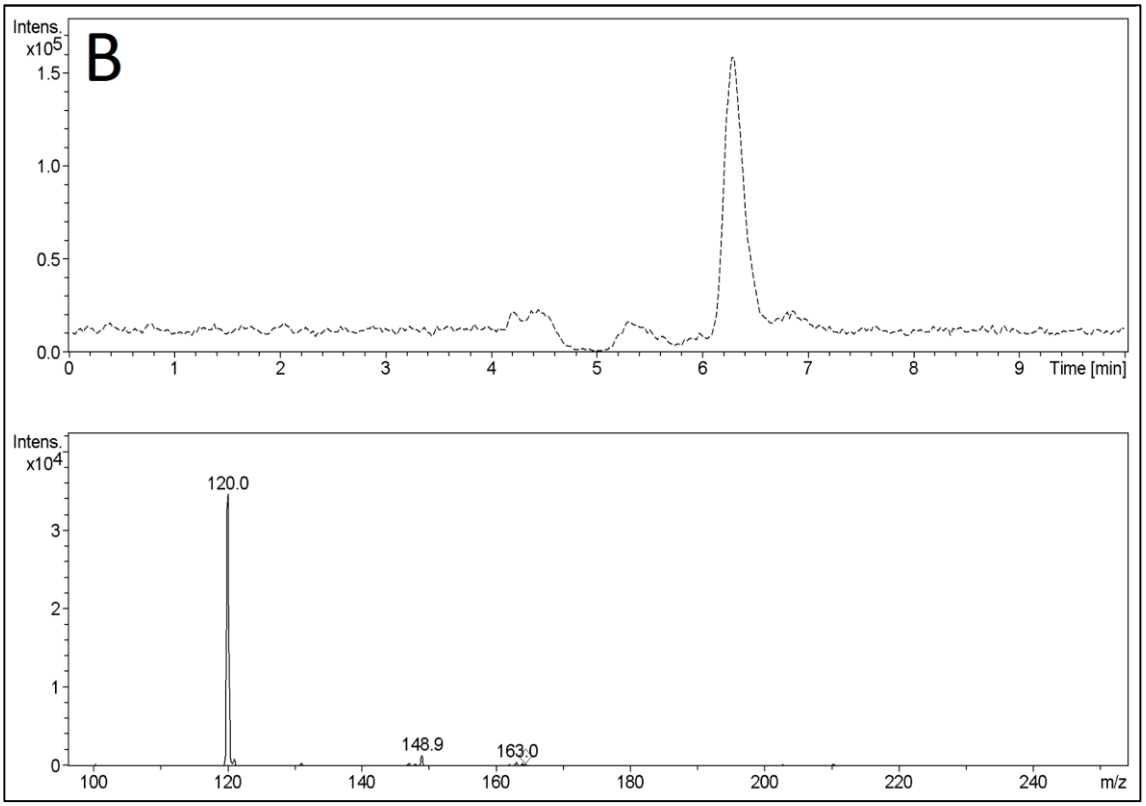


Figure 37: Representative chromatograms and spectra produced after Tandem MS. of ion mass=166 detected after incubation of mephedrone with pig liver microsomes
A: SIM mode and B: SRM mode.

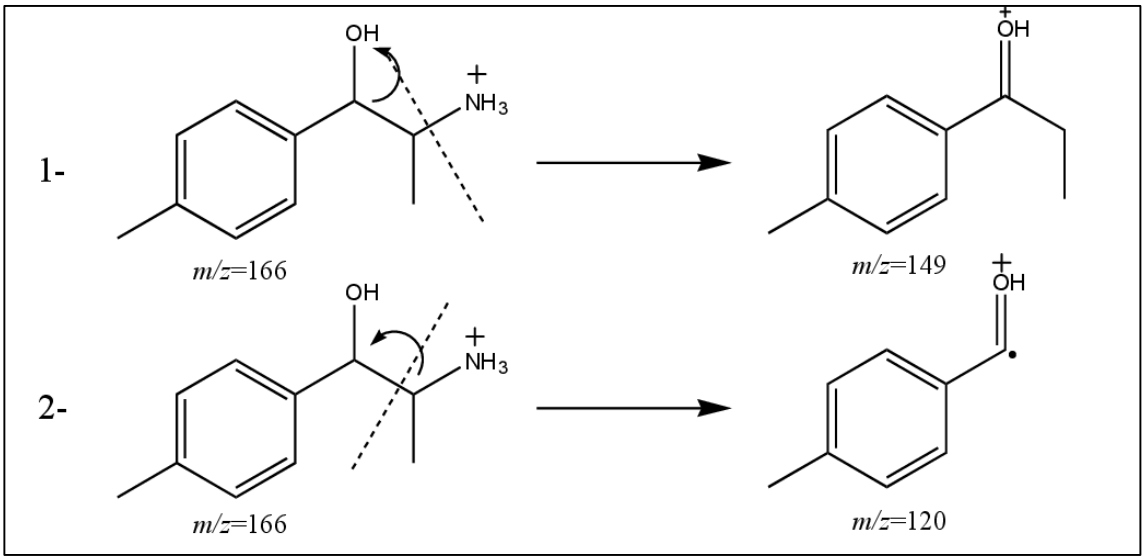


Figure 38: Proposed fragmentation pattern and mass spectrum of the metabolite ion of *m/z*=166 detected for the drug mephedrone after tandem MS utilizing LC-MS with proposed fragments suggestive of nor-dihydro-mephedrone

Suggestively, the proposed metabolic pathway of the detected metabolites is by hydroxylation of the methyl group attached to the aromatic ring for hydroxytolyl-mephedrone and a two-step N-demethylation and reduction of the ketone moiety two steps for nor-dihydro-mephedrone (Figure 39).

The detected metabolites in the current research work were described earlier by Meyer et al. and recently by Pedersen et.al. both *in vitro* and in *in vivo* studies. Meyer et.al. performed an *in vivo* study and reported some metabolites of mephedrone including the ones detected in the current research work, hydroxytolyl mephedrone and nor-dihydro-mephedrone. Pedersen et.al. performed *in vitro* studies utilizing infected insect cell microsomes, human liver microsomes and human liver S9 fraction. They also analysed forensic cases. Five metabolites of their expected metabolites list were detected including the one detected in current research work, hydroxytolyl mephedrone, while nor-dihydro-mephedrone was not detected neither *in vitro* or *in vivo* samples (Meyer et al., 2010; Pedersen et al., 2013). Compared to the metabolites described in current research work, two metabolites are described to be produced by *in vitro* metabolism: hydroxytolyl which was described earlier in both *in vitro* and *in vivo* studies, while nor-dihydro mephedrone was detected only *in vivo* study.

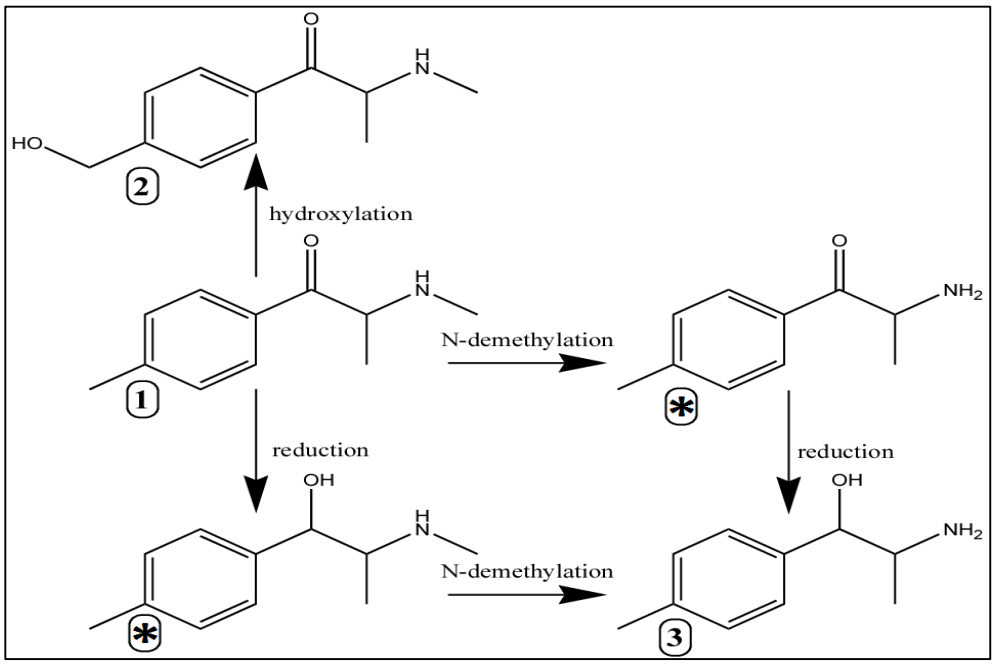


Figure 39: A proposed metabolic pathway through the identified metabolites of mephedrone utilizing LC-MS
(1) Mephedrone (2) hydroxytolyl-mephedrone (3) nor-dihydro mephedrone, (*) undetected intermediate

4.2.2. Methoxetamine

For methoxetamine, as discussed earlier, MXE is structurally similar to ketamine, which would suggest a similar metabolic pathway. Ketamine is metabolized through N-dealkylation to norketamine, followed by hydroxylation of norketamine at different locations and the formation of 5, 6-dehydronorketamine (Figure 40). The same metabolites were observed *in vitro* as well as *in vivo* in humans and animals' (Portmann et al., 2010; Schmitz et al., 2010).

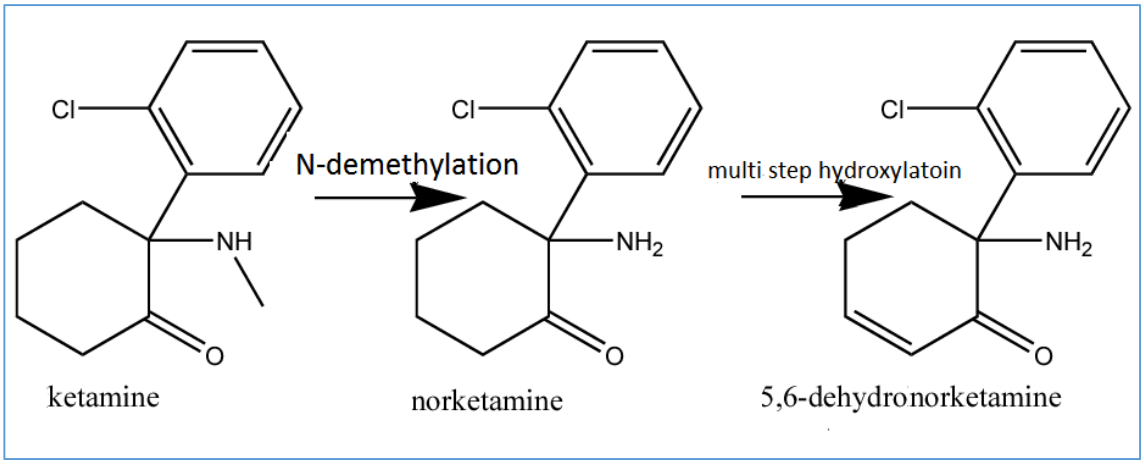


Figure 40: Summary metabolic pathway of ketamine

Meyer et al. recently utilised GC-MS for *in vivo* studies and detection of MXE metabolites in human and rat urine. They concluded that MXE may be metabolised through phase I and phase II enzymatic system. Phase I enzymatic reactions involves N-demethylation, O-demethylation and hydroxylation or a combination of these metabolic steps. It was concluded through their study also that phase II metabolic pathways are involved in the metabolism of MXE through sulphontaion or glucuronidation of most of these metabolites (Meyer et al., 2013). Menzies et al. performed *in vitro* studies utilizing LC-MS for the analysis of MXE metabolites after incubation with human liver microsomes. Phase I and phase II metabolites were detected in their study, where phase I metabolites were the product of N-demethylation, O-demethylation, reduction, hydroxylation and dehydrogenation.

Adapted from published data about MXE and similar drugs, a metabolic pathway can be proposed for phase I metabolism of MXE (Figure 41.). This proposed metabolic pathway would be used as

predictive tool for detection and analysis of metabolites by tracing specific *m/z* values and extraction the chromatograms for specific ion mass (Table 15)

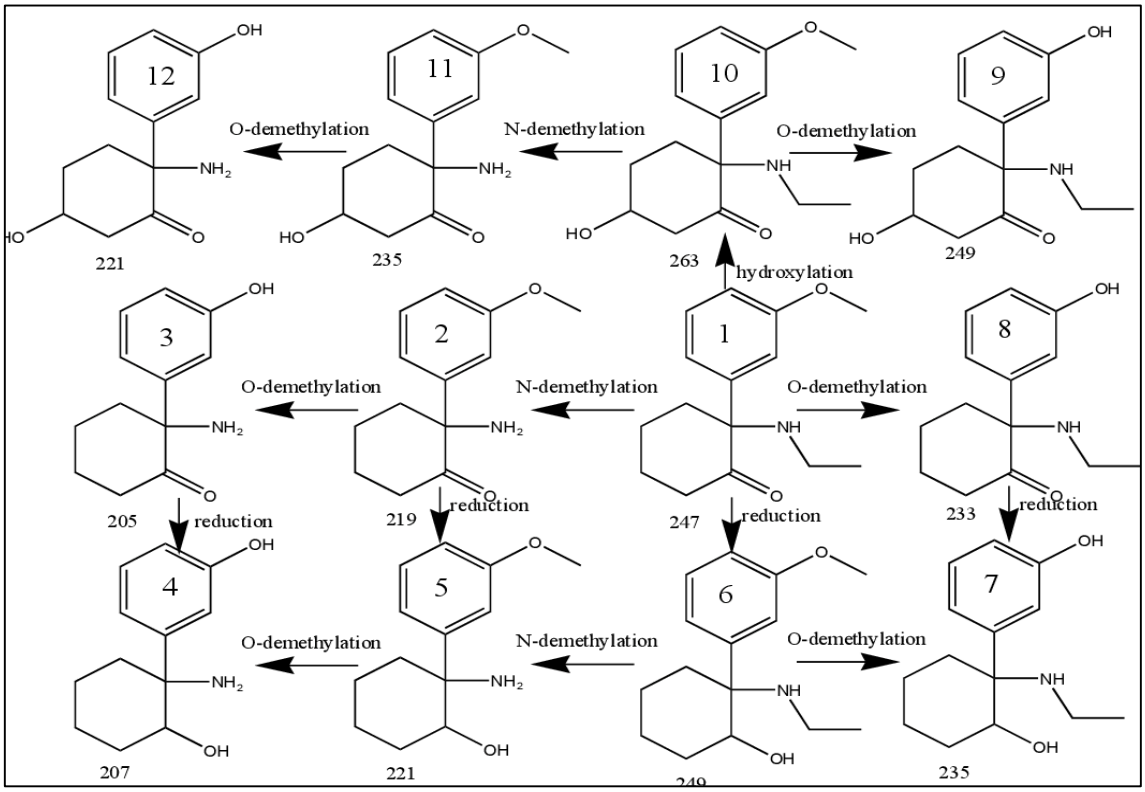


Figure 41: Suggested phase I metabolic pathway of MXE adapted from published data for the metabolism of MXE and similar drugs.

Table 15: Suggested metabolites produced from phase I metabolism of MXE after incubation with liver microsomes and analysis utilizing LC-MS/IOT and the monitored ion masses

Number[1]	**Name**	**MW[2]**	**Extracted MH^+**
1	MXE	247	248
2	nor-MXE	219	220
3	O-desmethyl-nor-MXE	205	206
4	O-desmethyl-dihydro-nor-MXE	207	208
5	Dihydro-nor-MXE	221	222
6	Dihydro-MXE	249	250
7	O-desmethyl- dihydro-MXE	235	236
8	O-desmethyl - MXE	233	234
9	O-desmethyl -hydroxy--MXE	249	250
10	hydroxy-MXE	263	264
11	hydroxy-nor-MXE	235	236
12	O-desmethyl-hydroxy- nor-MXE	221	222

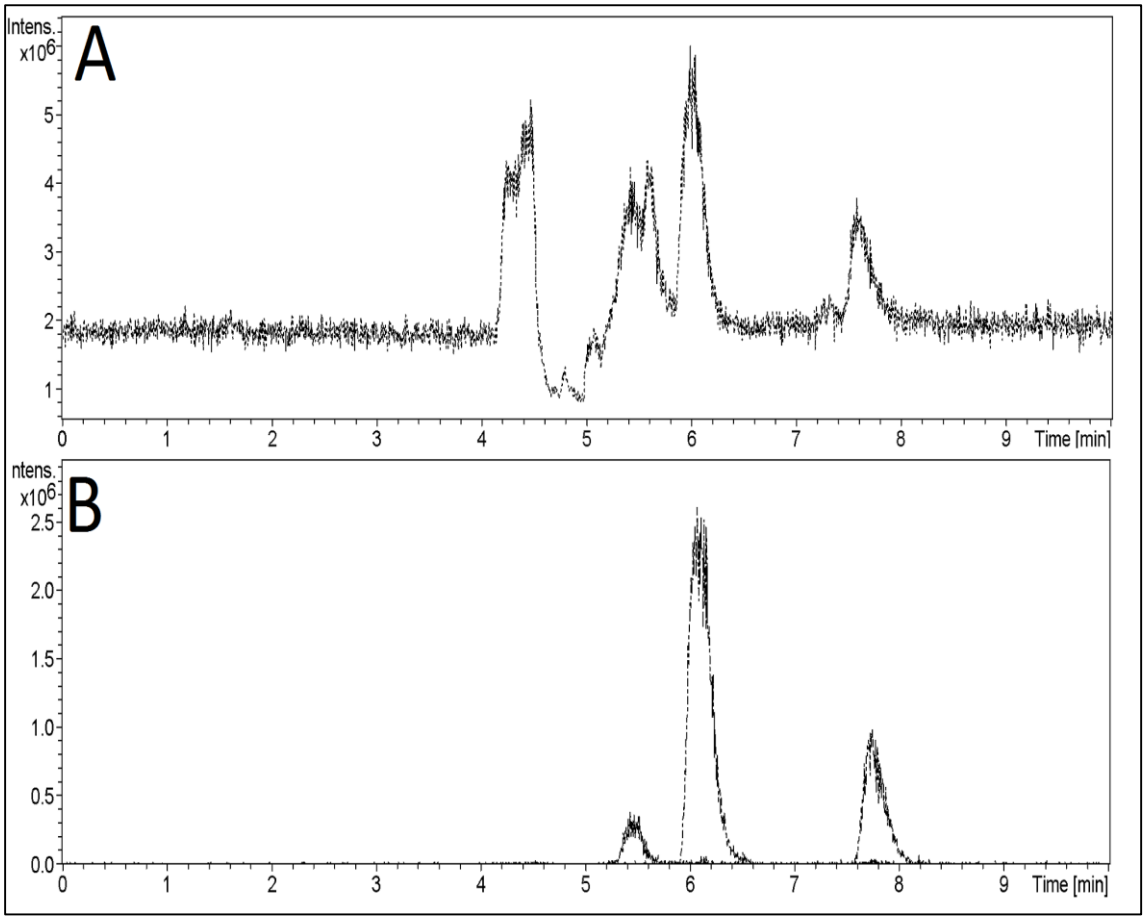
1: molecule number in reference to Figure 41 2: molecular weight
3: neutral loss of water 4: loss of hydroxyl

Total ion chromatogram(TIC) was extracted for the molecular ion of MXE and of each of the suggested metabolites. Extracted ion chromatograms (EIC) produced three peaks where one of the mass spectra is that of the parent drug MXE while the other two mass spectra were suggestive to be

one of the metabolites of MXE. The detected peaks that were suggestive to of the metabolites of MXE were having a spectrum with abundant MH^+=234 and MH^+=250 (Figure 42).

The detected peak with MH^+=248 appeared at 7.4 minute and has mass spectrum with abundant MH+=248 and secondary fragments of *m/z*= 203 and *m/z*=175, similar to the observed mass spectrum of MXE control runs., which confirm the identity of the peak to be of MXE (see Figure 30)

The detected peak with MH^+=234 appeared at 6.1 minute and has mass spectrum with abundant MH+=234 and secondary fragments of *m/z*= 189, which are less 14 units than the respective ion masses observed in mass spectrum of MXE of *m/z*=248 and *m/z*=203. The detected peak with MH^+=250 appeared at 6.3 minute and has mass spectrum with abundant MH+=250 and secondary fragment of *m/z*=205. Following the suggested metabolic pathway, it is proposed that the produced metabolite with MH+=234 is O-dimethyl MXE, while the other with MH+ =250 is either dihydro-MXE or O-desmethyl -hydroxy-MXE (Figure 43).



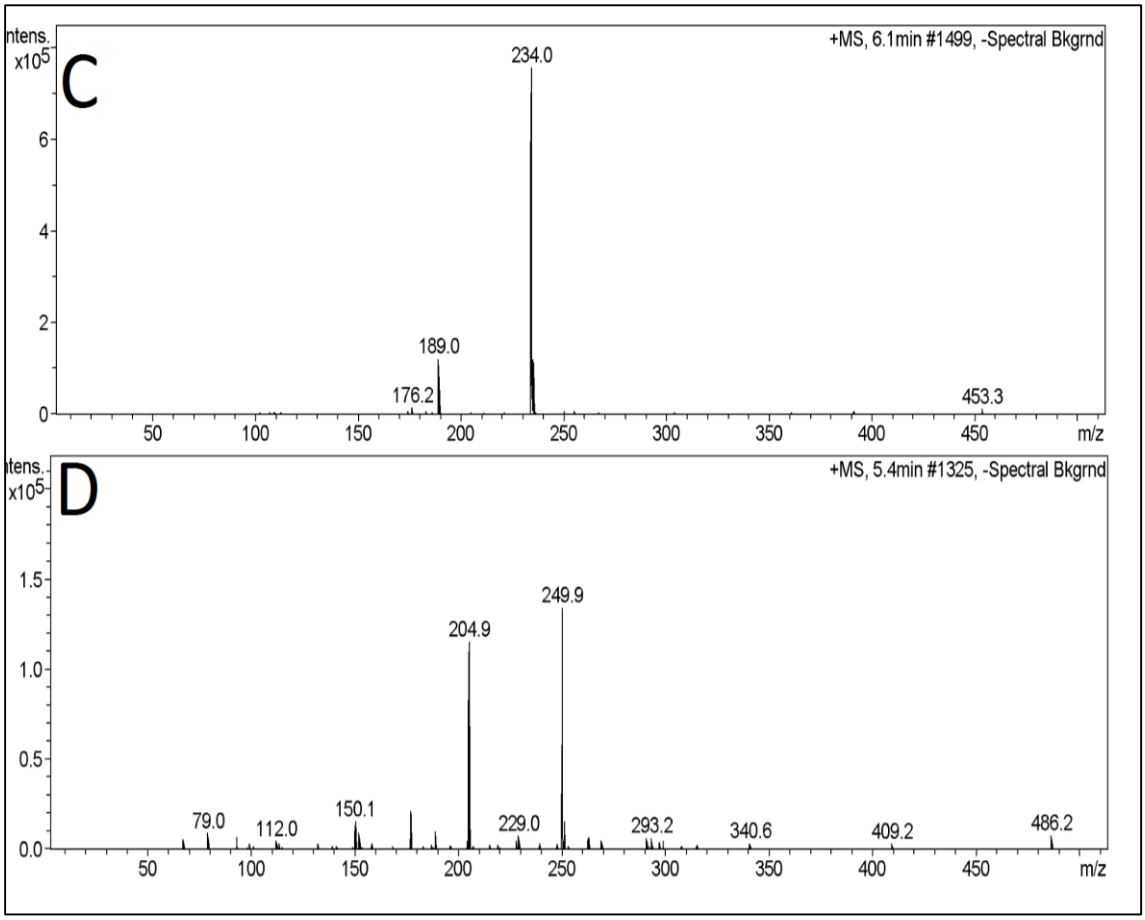


Figure 42: Representative chromatograms and spectra of the detected peaks suggestive of MXE metabolites after incubation with pig liver microsomes
A: TIC, B: EIC and C: mass spectrum of detected peak with MH^+=234 and D: mass spectrum of detected peak with MH^+= 250.

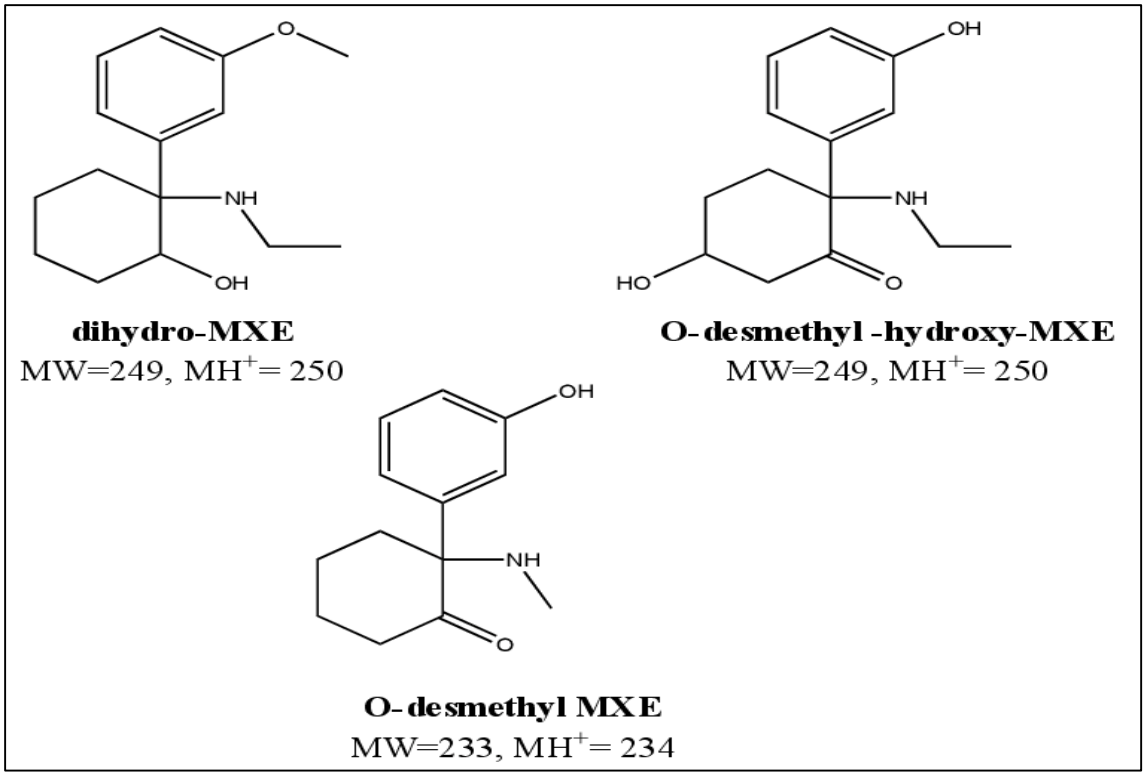
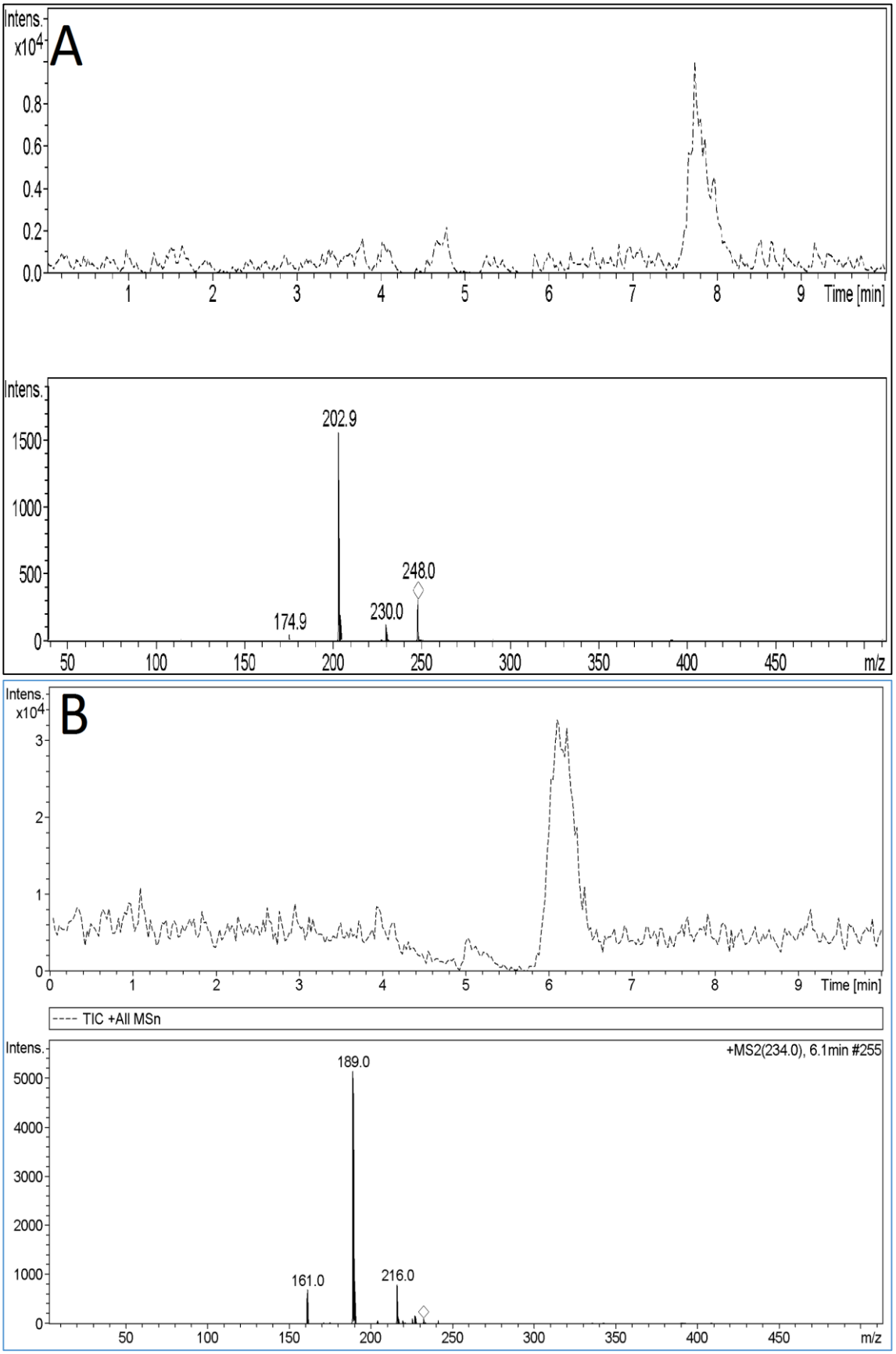


Figure 43: proposed metabolites of the identified peaks for the drug MXE utilizing LC-MS after incubation with in-house prepared pig liver microsomes

Samples were analysed through MS/MS analysis using selected reaction monitoring modes The selected ions were those of the molecular ion of MXE (MH+=248) and of the suggested metabolites (MH+= 250 and MH+=234). Analysis of the samples in SRM mode with selection of ion mass=248 produced a chromatogram with one representative peak at 7.3 min, where its mass spectrum contains the fragments of *m/z*= 203 and *m/z*=175 similar to the one previously observed in MS analysis of MXE. A new fragment of *m/z*=230 was detected which is 18 units less than the molecular ion, suggestive it is due to neutral loss of water. Analysis of the samples in SRM mode with selection of ion mass=234 produced a chromatogram with one representative peak at 6.1 min, where its mass spectrum contains the fragments of *m/z*= 189 and *m/z*=161. Those observed fragments are similar to the one previously observed in MS analysis of the suggested metabolite and comparable to the ones observed through MS analysis of MXE. The observed fragments are 14 units less than the respective ones observed for MXE. Similar to the observed fragment in case of MXE, a new fragment was detected of *m/z*=216 which is 18 units less than the molecular ion of the suggested metabolite. Analysis of the samples in SRM mode with selection of ion mass=250 produced a chromatogram with low intensity signal. Tracing TIC at the expected retention time of 5.4 min for the suggested metabolite of MH+=250 one representative peak at 5.4 min showed a mass spectrum with one fragment of *m/z*=205. The molecular ion of the suggested metabolite is two units more than the one of MXE, and the observed fragment of *m/z*=205 is comparable to the respective one observed for MXE of *m/z*=203.which is also two units more (Figure 44).

The mass spectra of the MXE and proposed metabolites showed similar fragmentation pattern between each other and in compare with tandem MS data. A proposed fragmentation pattern of the drug MXE and comparison with the detected fragments of the suggested metabolites may confirm the identity of each detected molecule. Proposed fragmentation pattern of MXE from the identified fragment after tandem MS analysis of ion mass=248 shows that the fragment of *m/z*= 203 is a product of net loss of ethyl-amino group, the fragment of *m/z*=230 is a product of net loss of water and the fragment of *m/z*= 175 is a product of α-cleavage followed by net loss of (C_4H_8O). Another proposed fragmentation pathway to produce the fragment of *m/z*=203 as by α-cleavage followed by net loss of

(C_2H_4O) that will fragment further to produce the fragment of *m/z*=175. Other proposed fragments were not detected (Figure 45).



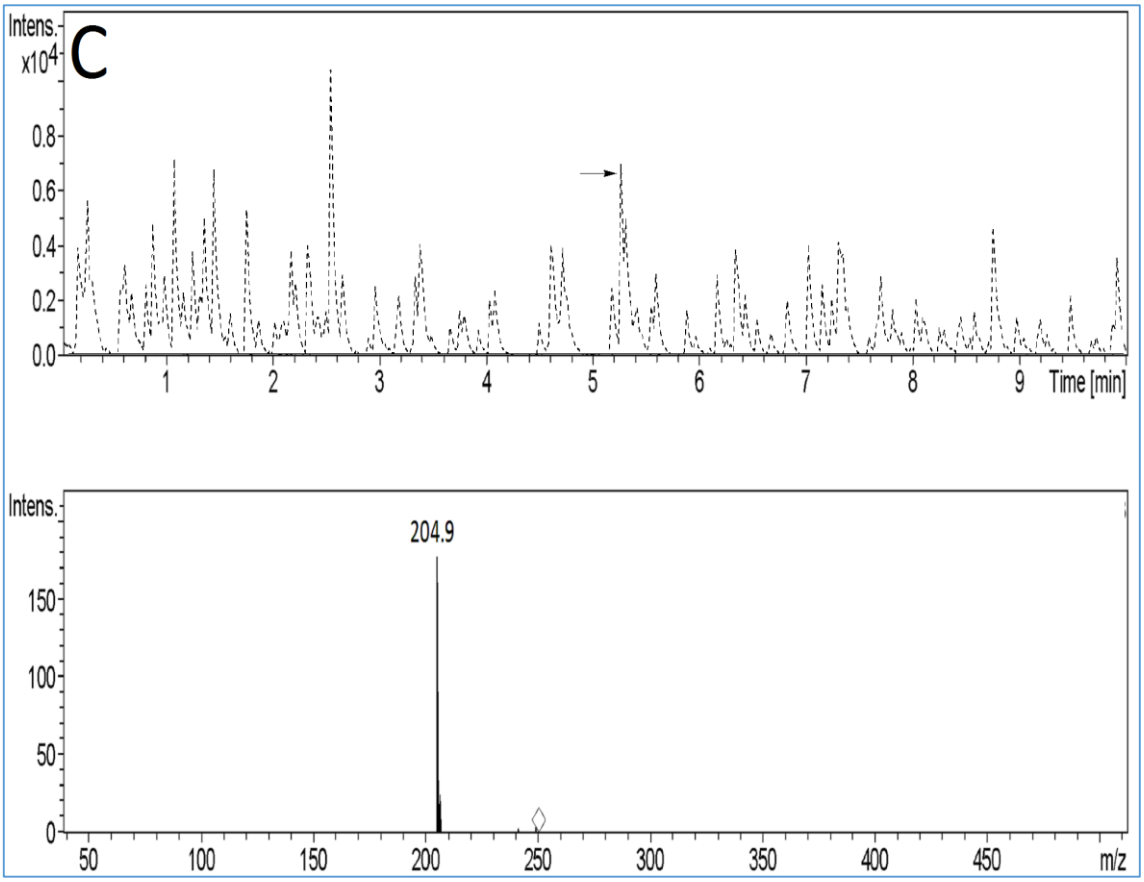


Figure 44: Representative chromatograms and spectra produced after Tandem MS. of selected ion masses detected in EIC after incubation of MXE with pig liver microsomes
A: MH+=248, B: MH+=234 and C: MH+=250

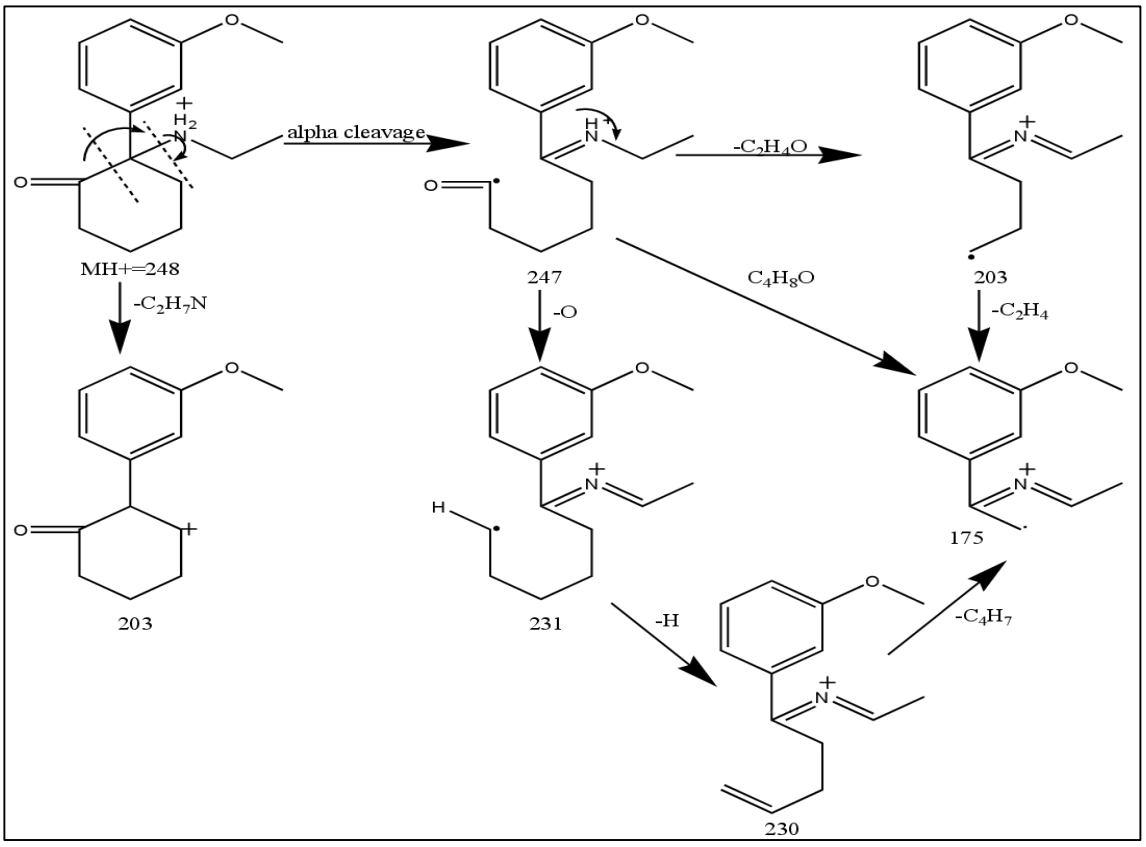


Figure 45: proposed fragmentation pattern of MXE through the detected fragment after MS/MS analysis

Similarly, and in comparison to the proposed fragmentation pathway of MXE, the identity of the detected metabolite of MH+=234 could be confirmed as O-desmethyl-MXE. Similar fragmentation pathway is observed with 14 units less due to O-demethylation (Figure 46). For the detected peak with M+=250, two metabolites are suggested. Low intensity signal was recorded and the suggested fragmentation pathways of the suggested metabolites were not conclusive for the identity of the molecule. The proposed fragmentation pathway of the dihydro-MXE in respect to the one proposed and observed for MXE produce a fragment of *m/z*=205 through different proposed pathway.

The other suggested metabolite with MH+=250 was O-desmethyl –hydroxy-MXE. Hydroxylation of the cyclohexanone moiety can occur in any site. However, compared to the metabolism of the structurally related ketamine, and for the purpose of description, it was assumed hydroxylation will take place on the 5 or 6 position. None of the other proposed fragments were detected (Figure 47).

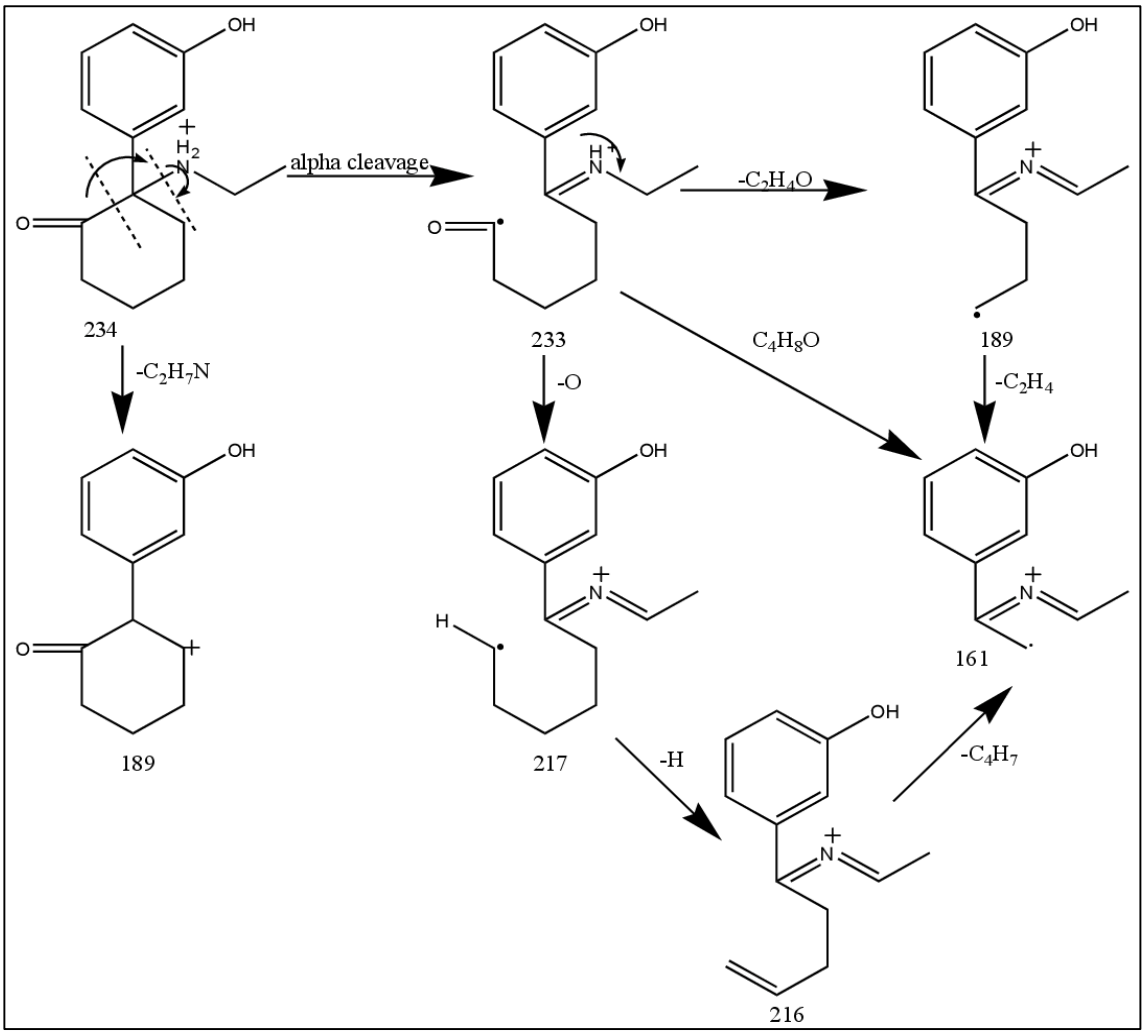


Figure 46: proposed fragmentation pattern of O-desmethyl-MXE in respect to the proposed fragmentation of MXE after MS/MS analysis

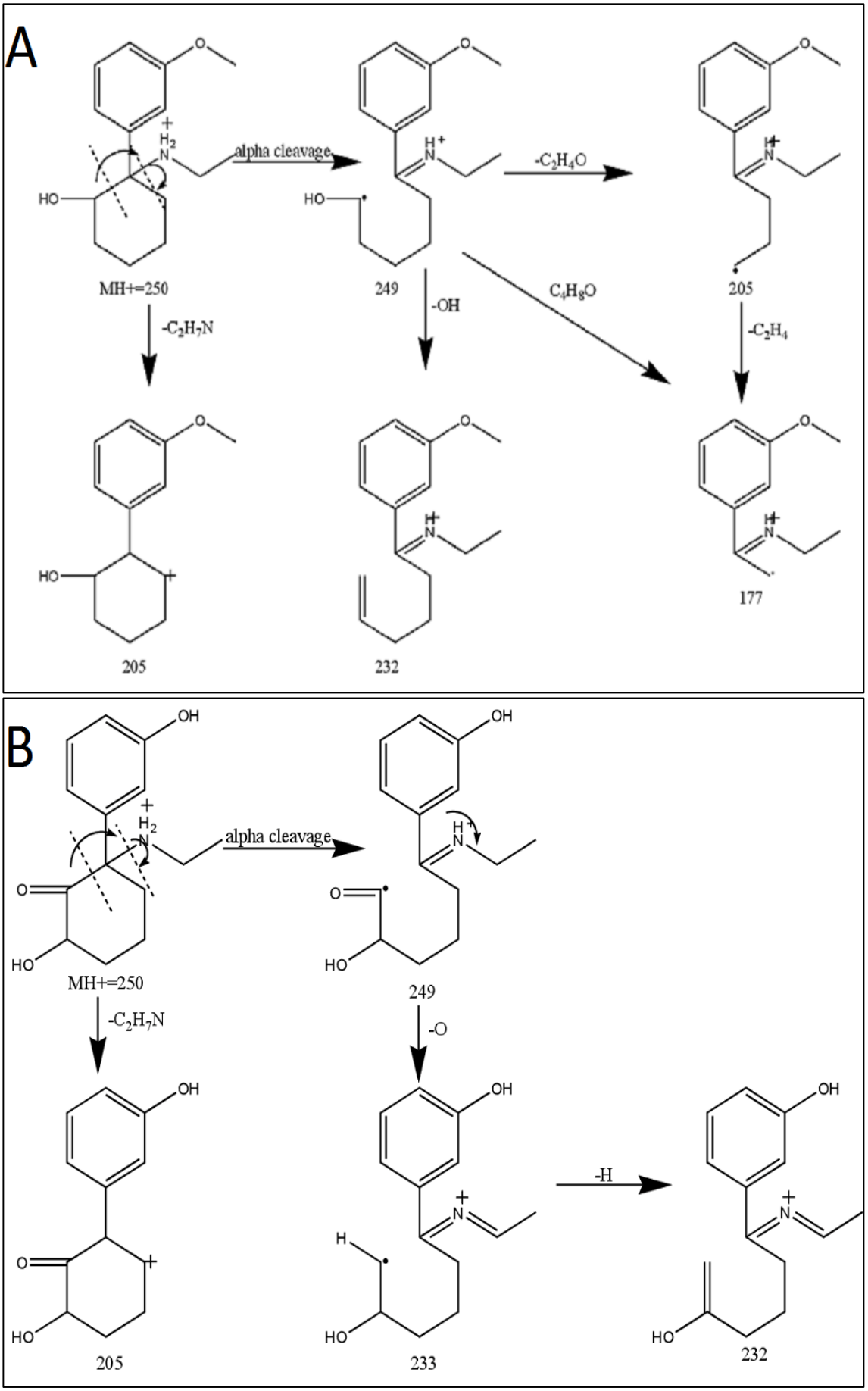


Figure 47: proposed fragmentation pattern of O-desmethyl–hydroxy-MXE in respect to the proposed fragmentation of MXE after MS/MS analysis

No other metabolites were identified that supported the initial proposal that methoxetamine has a similar metabolic pathway to ketamine – i.e. hydroxylation and N-dealkylation. However, the proposed O-demethylation pathway for methoxetamine is similar to other metabolic pathways described by *in vitro* studies of other drugs with a similar methoxy functional group attached to the benzene ring. Tramadol is a typical example of drugs with very similar structure compared to methoxetamine with peripheral methoxy group attached to the benzene ring (Sevcik et al., 1993). The major metabolite in the case of tramadol is by O-demethylation to produce O-desmethyltramadol, the active metabolic product of the tramadol (Figure 48).

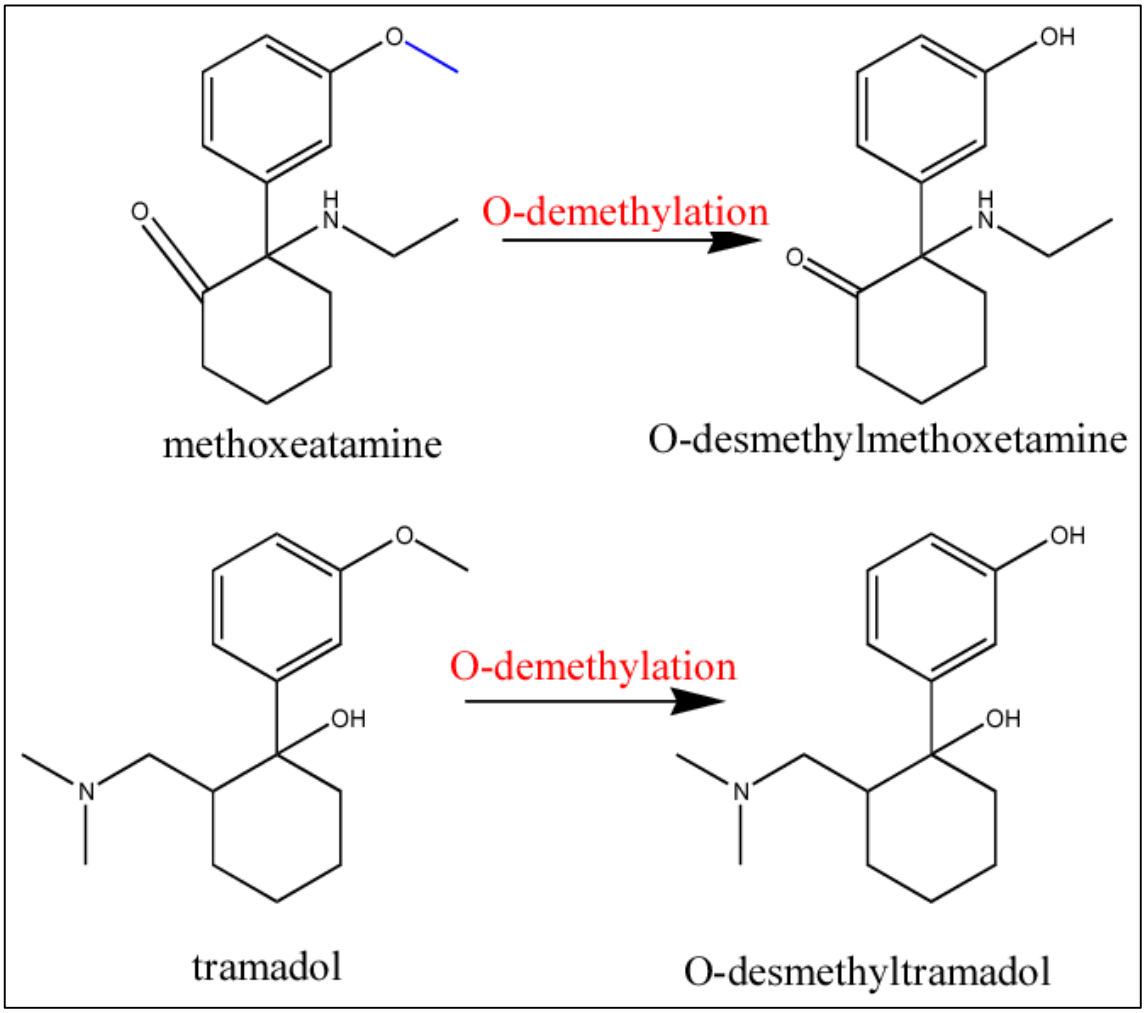


Figure 48: Comparison between the structure of MXE and tramadol showing similarity in structure and metabolic pathway by O-demethylation

4.2.3. Methcathinone

To the best of knowledge, limited data are available about the metabolism of methcathinone. However, methcathinone, theoretically, could be metabolised into cathinone. (Sorensen, 2011). Very recent in 2015, Chen published data about *in vitro* study of metabolism of designer drugs including methcathinone and cathinone was reported as a metabolite for methcathinone through N-demethylation. (Chen, 2015).

Compared to mephedrone and depending on the theoretical demethylation of methcathinone, a metabolic pathway can be proposed for methcathinone where it could be metabolised into nor-methcathinone and dihydro-methcathinone by demethylation and reduction pathway, respectively. Another metabolite is possible by combination of these pathways to produce nor-dihydro-methcathinone (Figure 49)

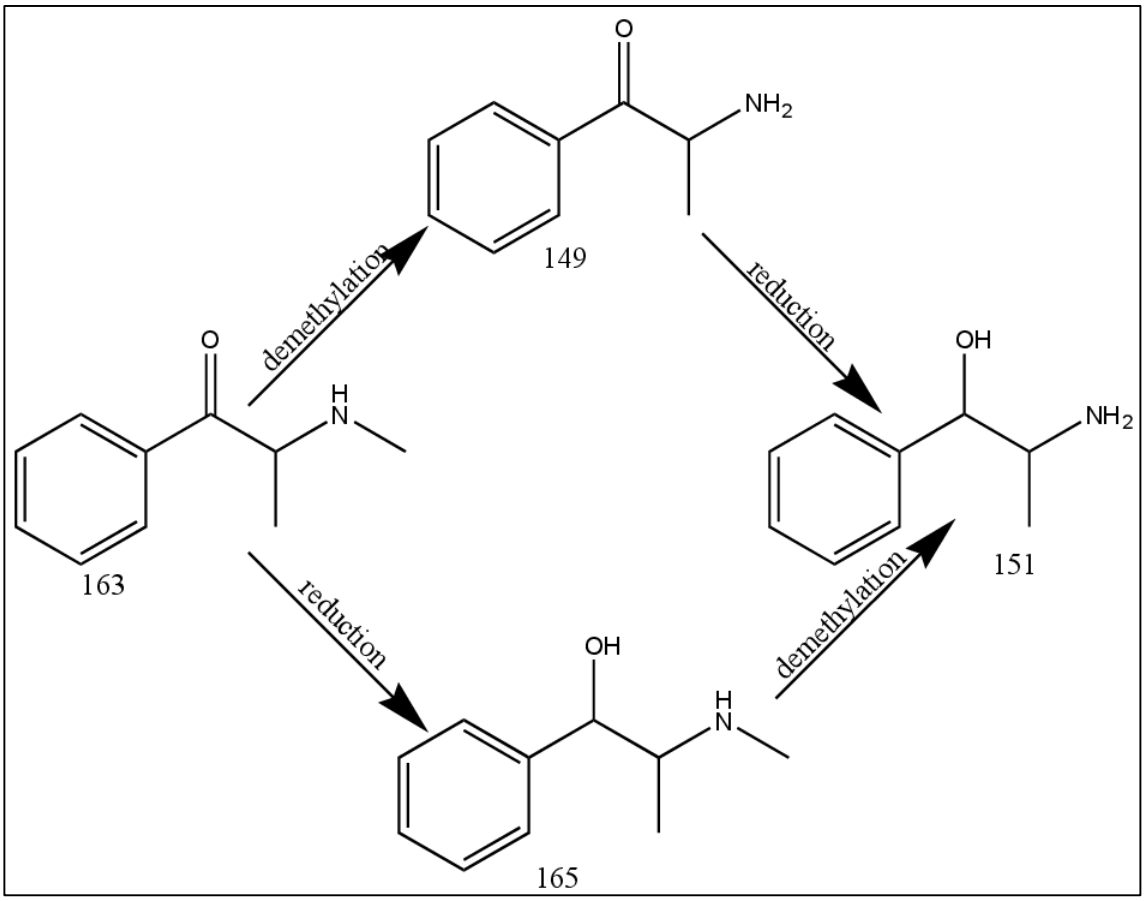


Figure 49: suggested metabolic pathway of methcathinone

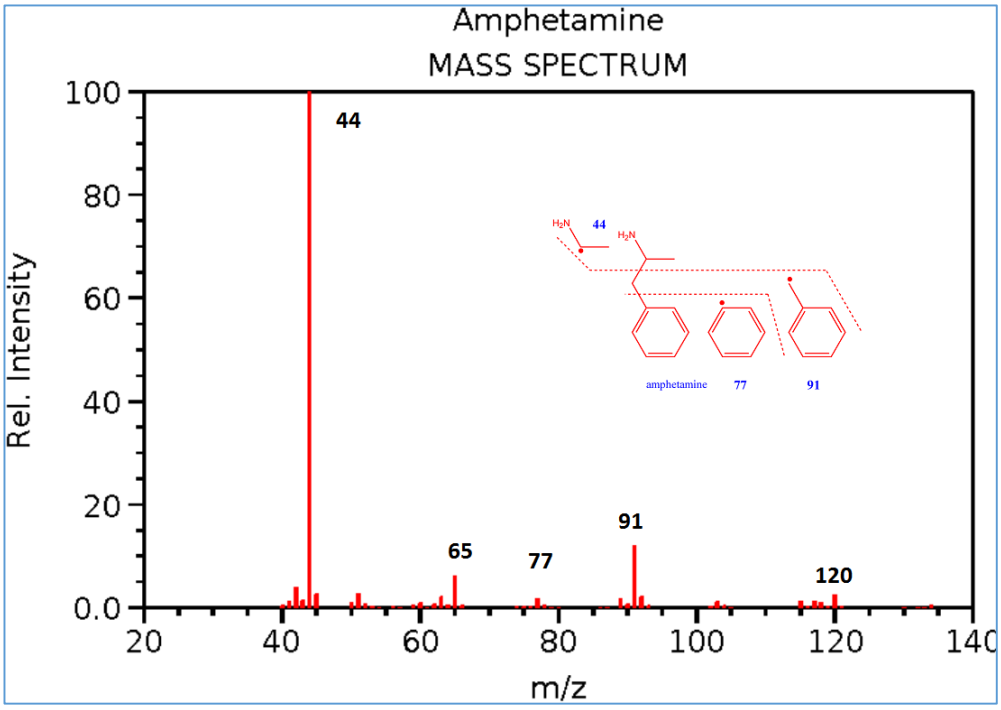
Total ion chromatograms were extracted for the following molecular ion of the parent drug methcathinone and of the suggested metabolites, i.e.: 164, 150, 166 and 152. It was possible to detect the peaks that correspond to the parent drug. However, it was not possible to identify any peak for the molecular ion of the suggested metabolites.

4.3. *In vitro* studies of selected NPS using hepatocytes and analysis utilizing GC-MS

4.3.1. analysis of the selected NPS utilizing GC-MS

The aim of this part of the work is to determine retention times' and the fragmentation pattern of selected NPS utilizing GC-MS as fingerprint for each drug. The results were obtained by preparing solutions of NPSs of interest. Mephedrone (4MMC), methcathinone (MCAT), 4-fluoromethamphetamine (4FMA) and methoxetamine (MXE) were prepared in acetonitrile and injected onto the GC-MS system. The retention times, under the specified GC-MS conditions, were identified for 4MMC, MCAT, 4FMA and MXE as 7.72, 6.79 5.57 10.41 (± 0.05) minutes respectively. The drugs were then derivatized using BSTFA and injected onto the GC-MS system, and the produced mass spectra were compared to those of underivatized drugs**.**

The drugs 4MMC, MCAT and 4FMA are structurally similar to cathinone and amphetamine. By comparison of spectral data of these drugs with each other, and following the general fragmentation rules, a fragmentation pattern can be expected for each of these drugs. The mass spectra of amphetamine and cathinone show the *m/z*=44 as the base peak, the mass of the peripheral ethylamine group, a product of α–cleavage, one of the favourable fragmentation schemes. (Figure 50)



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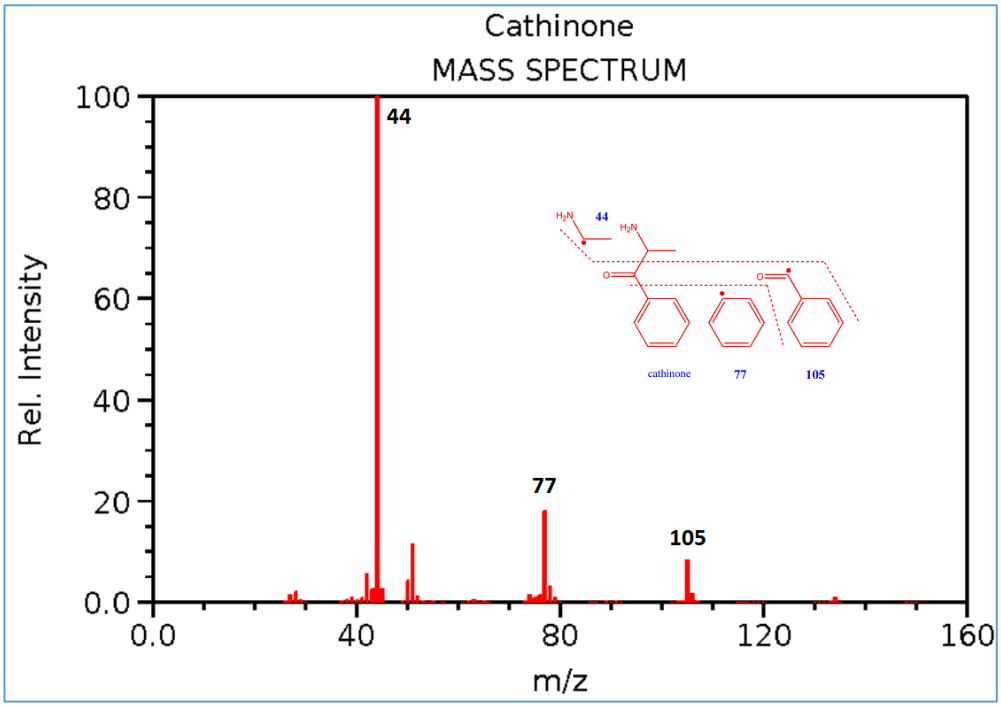


Figure 50: Mass spectra of the amphetamine and cathinone
Spectral data source: NIST Chemistry WebBook (**http://webbook.nist.gov/chemistry**).
Mass labels and fragmentation pattern added for clarification.

4.3.1.1. Mephedrone

For mephedrone, standards were injected onto the GC-MS, and resultant mass spectra were analysed. Two adjacent peaks were detected, the first peak eluted at 7.72 minutes while the second at 7.80 minutes (Figure 51).

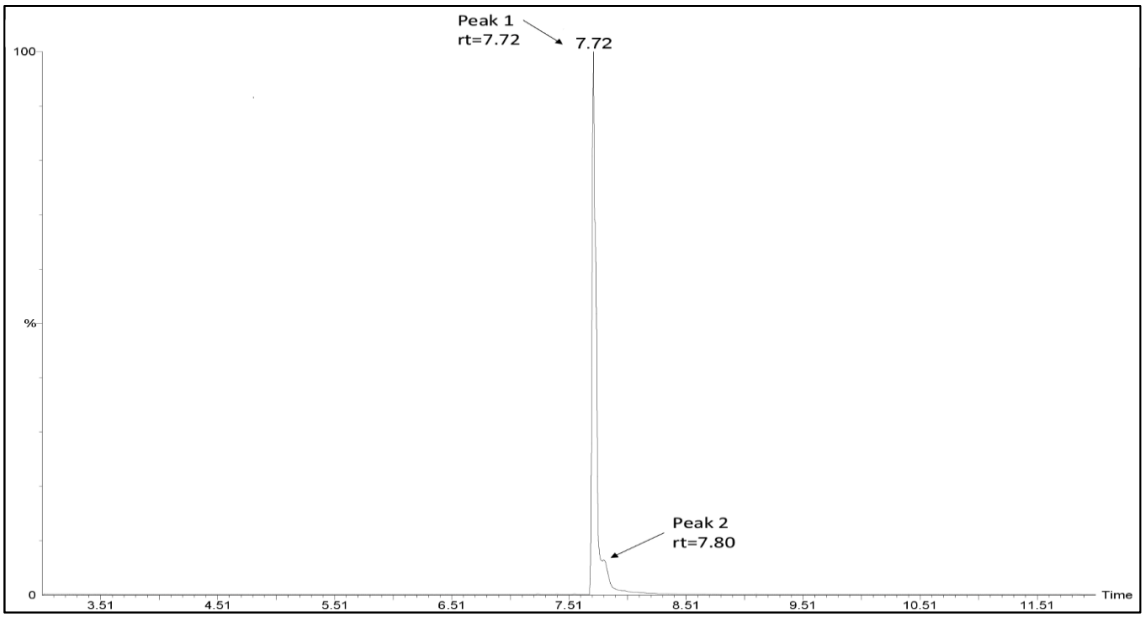


Figure 51: Chromatogram produced after injection of mephedrone onto GC-MS system

Mephedrone is structurally similar to cathinone, both having an (± alkyl) amine group, β-ketone moiety and peripheral (±methyl) phenyl moiety. The fragmentation pattern of mephedrone is expected to follow the same fragmentation pattern of cathinone, where it will show base peak of *m/z*=58 in compare to the base peak of *m/z*= 44 due to the presence of extra amine-attached-methyl group. Due to the extra methyl group attached to the phenyl moiety in mephedrone, secondary peaks of *m/z*=77 and *m/z*= 105 in case of cathinone will be shown as mass peaks *m/z*= 91 and *m/z*=119 (Figure 52).

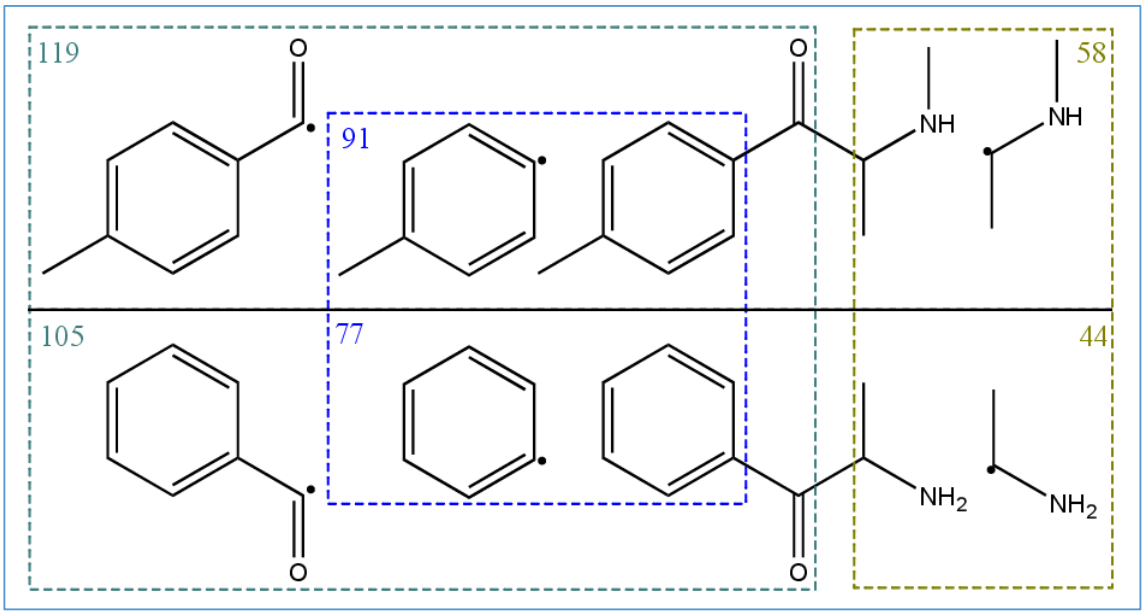


Figure 52: fragmentation pattern of mephedrone (upper) in compare with fragmentation pattern of cathinone (lower).

Mass spectrum of the first detected peak showed the molecular ion with *m/z* 177 in addition to *m/z* values of 162, 147, 119, 91 and 58. The base peak was *m/z* 58 that corresponds to the peripheral ethyl-methyl-amine group, a product of α-cleavage (Figure 53).

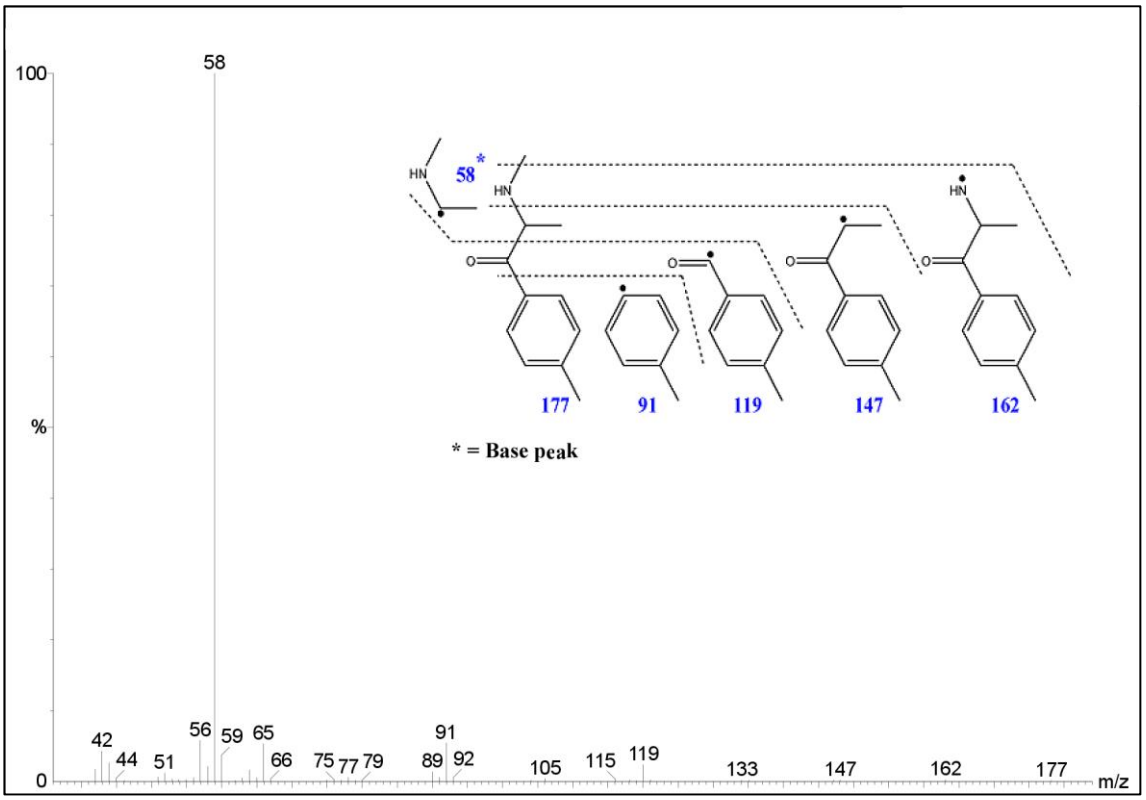


Figure 53: Mass spectrum of the first detected peak after injection of mephedrone onto GC-MS system and the proposed fragmentation pattern

The relative abundance of an '*m/z* value' and its corresponding isotope were checked against their expected natural occurrence, another confirmative tool for the identity of an analyte (Baron et al., 2011). The corresponding isotope for the *m/z* values of 147, 162 and 177 were not detected, which could be due to the low relative abundance of these *m/z* values in the produced GC-MS spectrum of less than 0.2%

Table 16: Natural occurrence and relative abundance of *m/z* value and its corresponding isotope for mephedrone.

***m/z* vs isotope**	**Natural Occurrence**	**Relative Abundance**
58 vs 59	96.6% vs 3.4%	96.5 vs 3.5 %
91 vs 92	92.7% vs 7.3%	92.2% vs 7.8%
119 vs 120	91.8% vs 8.2%	91.4% vs 8.6%
147 vs 148	90.0% vs 10.0%	ND[1]
162 vs 163	89.8% vs 10.2%	ND
177 vs 178	88.9% vs 11.1%	ND

1: Not detected

The mass spectrum of the second detected peak of mephedrone showed *m/z* values with a different but comparable pattern to the one noticed in the mass spectrum of the first detected peak. The noticed pattern was the appearance of *m/z* values 175, 160 and 56 as the base peak, which are less two mass units of the corresponding *m/z* values =177, 162 and 58 respectively. The other *m/z* values, i.e. *m/z*= 119 and 91 are appearing in both spectra. The noticed pattern is affecting the fragments containing the amine group while not affecting the fragments containing the phenyl group which may conclude that the fewer mass units is affecting the part of the structure containing the amine group. A possible explanation for the source of this additional peak is that it is an impurity, by-product or mephedrone related molecule during synthesis. Another possible explanation for the appearance of these less two mass units' fragments is the effect of pyrolysis processes upon injection (Zuba, 2012). Camillery et.al. described the observation of *m/z*=56, when analysing mephedrone utilizing GC-MS, as a minor ion caused by the loss of H_2. Also, they described the observation of *m/z*= 42 as a minor ion also caused by the loss of CH_4 (Camilleri et al., 2010). Further work is needed to confirm the identify this less two unit's molecule (Figure 54)

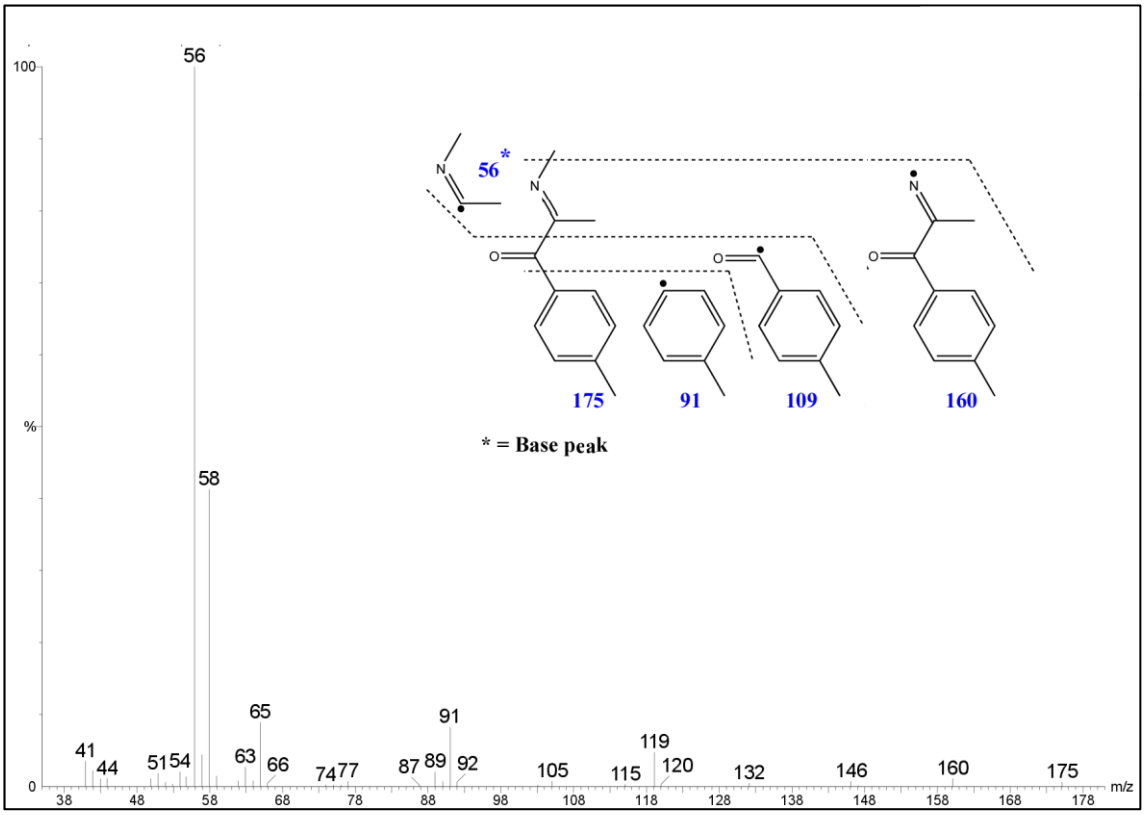


Figure 54: Mass spectrum of the second detected peak after injection of mephedrone onto GC-MS system and the proposed fragmentation pattern

After derivatization, the chromatogram showed four identifiable peaks: 7.69, 7.81, 7.89 and 8.84 minutes, where the mass spectra of the first two peaks was comparable to those previously detected for the underivatized mephedrone, while the other two are suggestive of the derivatized mephedrone (Figure 55).

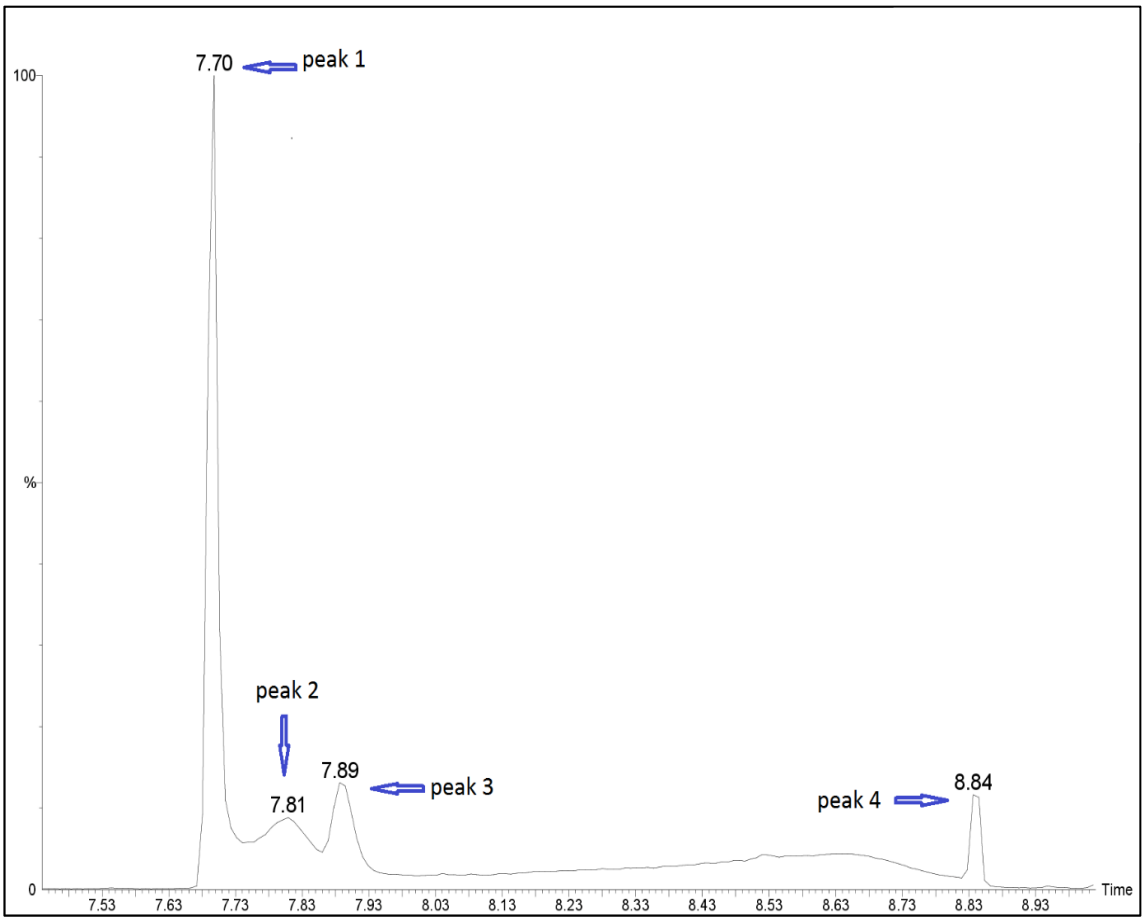


Figure 55: Chromatogram recorded for mephedrone after derivatization and injection onto GC-MS system

As expected from the molecular structure of mephedrone, the silylation reaction will involve the replacement of available active hydrogen of the amine functional group with a trimethylsilyl group (Orata, 2012). Mass spectra of each peak was analysed to compare the masses to each other, where it shows that the mass spectra of detected peaks 3 and 4 were both having comparable mass spectra to each other and to the ones of underivatized drug. Successful derivatization with BSTFA, and depending on number of attacked functional group, is expected to produce comparable fragments of the parent molecule plus the mass of one or more of trimethylsilyl functional group, i.e. plus 72, 144...etc.

Mass spectrum of peak 4 showed mass spectra comparable with the one of peak 1, where the base peak was of *m/z*= 130 (58+72), corresponding to the base peak of underivatized mephedrone of *m/z*=58, which is produced by the replacement of the available active hydrogen of the amine group by the trimethylsilyl functional group (Figure 56).

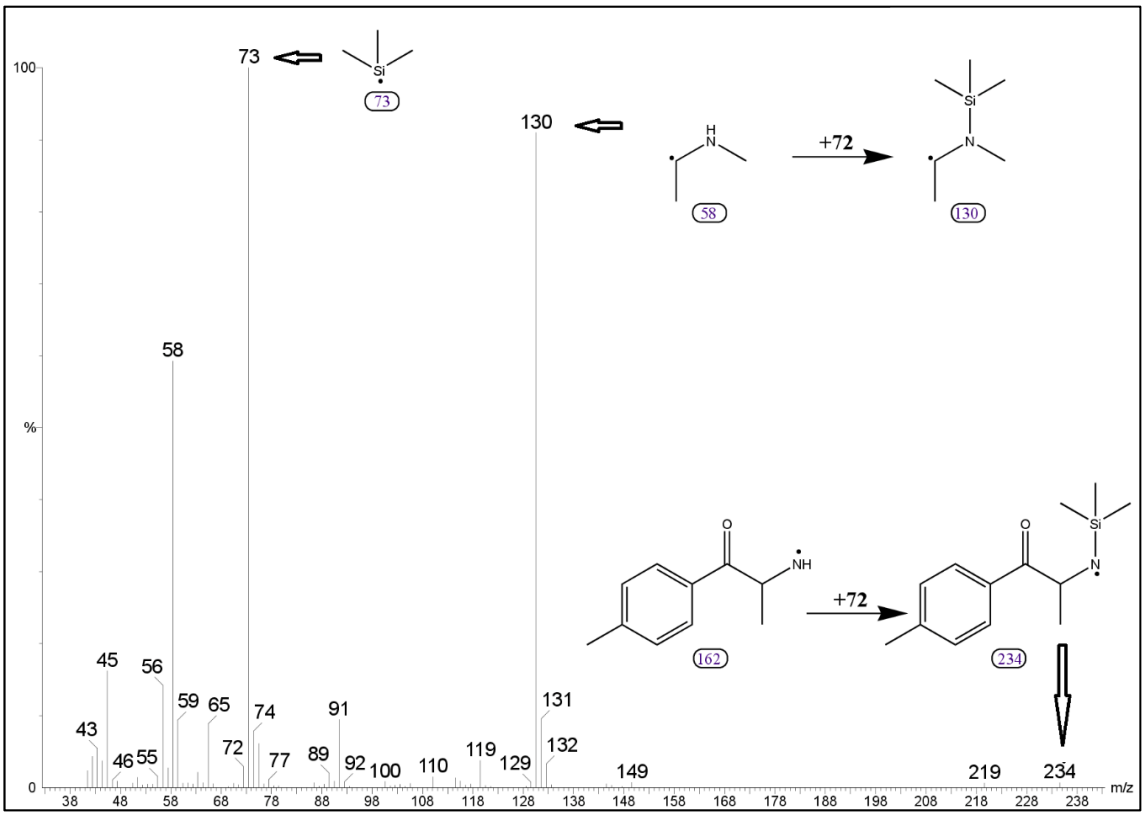


Figure 56: Mass spectrum of detected peak 4 of derivatized mephedrone after injection onto GC-MS system

Mass spectrum of peak 3 also showed comparable pattern with that of other peaks, but with the absence of the expected base peak *m/z*=130. A possible explanation is that the derivatization reaction took place on the enol form mephedrone, replacing the active hydrogen of the hydroxyl group by the trimethylsilyl functional group. This appears to be the most satisfactory explanation as the expected base peak of *m/z*=130 that was produced by replacement of active hydrogen of the amine group was not detected here in peak 3. In general, the derivatization reaction preferably took place on hydroxyl group of the enol form of the molecules, though the availability of active hydrogen on the amine group as the reactivity of hydroxyl functional group toward silylation is much more than the reactivity of amines (Orata, 2012). However, mass spectra of peak 3 showed base peak of *m/z*=56, same as noticed in the other peak detected in the chromatogram of underivatized mephedrone.

Silylation reactions may produce unexpected artefacts when reacting with some functional groups such as ketones and aldehydes, as was described by Little, who suggested the formation of trimethylsilyl-enol molecules for ketones with α-hydrogen (Little, 1999). In fact, the derivatization of the enol form of ketones is usually considered more troublesome rather than a target, which mandate to drive the keto-enol equilibrium toward the ketone or non-enolisable derivatives. However, some studies suggested the preference of silylation of the enol form of the ketone, a reaction that take place in one-step, as this derivative will elute earlier than the methoxime derivative. (Novotny and Wiesler, 1984). This supports the proposal that peak 3 detected for the derivatized mephedrone is the product of derivatization of the enol form of mephedrone, as it eluted earlier than peak 4, which is proposed to be the derivatized form of the ketone form of mephedrone. Another important mass value, *m/z*= 56, was detected as base peak in the mass spectra of peak 3 of derivatized mephedrone, which is comparable to the base peak detected in mass spectra of peak 2 of underivatized mephedrone (see Figure 54). This is most probably due to the migration of the double C-C bond of the enol from of mephedrone to produce double N-C bond, a possible mechanism during GC-MS-ES analysis.

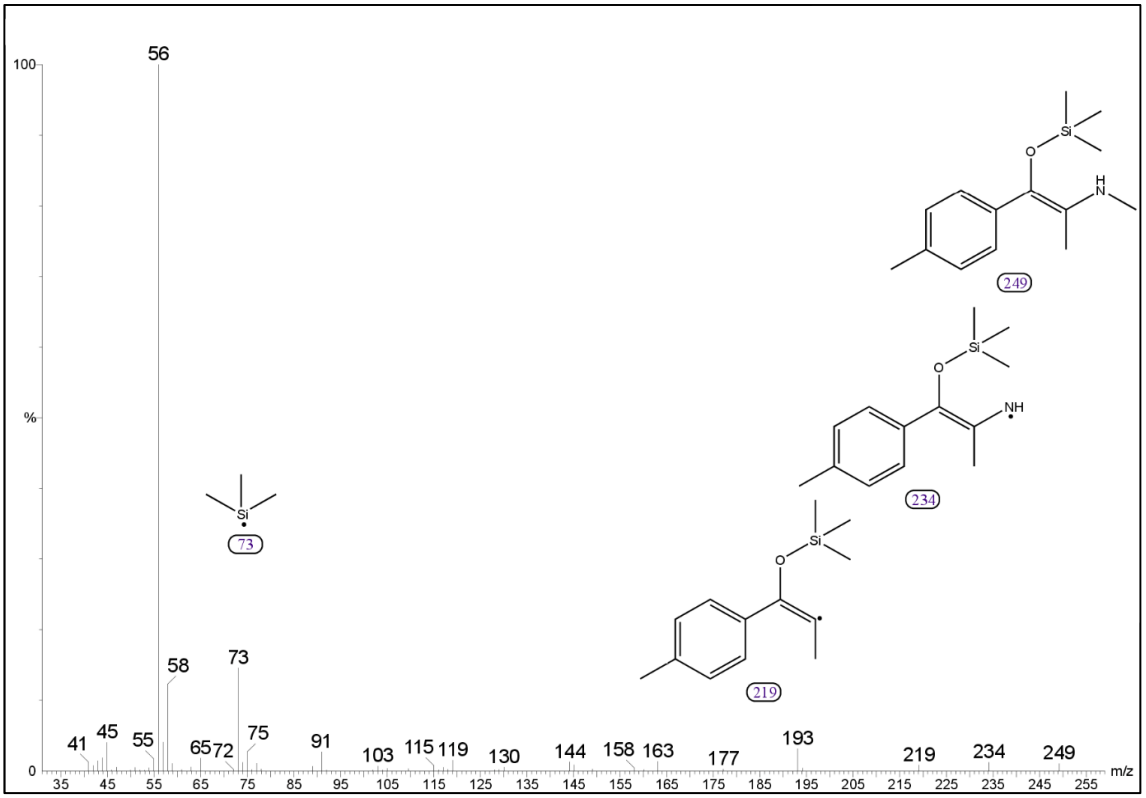


Figure 57: Mass spectrum of detected peak 3 of derivatized mephedrone after injection onto GC-MS system

4.3.1.2. Methcathinone

Applying the same principles followed earlier, the fragmentation pattern of methcathinone was studied in this part of the work. Methcathinone is structurally similar to cathinone and mephedrone, as mephedrone is the methylated form of methcathinone that in turn is the methylated form of cathinone

Conclusively, the fragmentation pattern of mephedrone is expected to follow the same fragmentation pattern of cathinone, where it will show base peak of *m/z*=58 in compare to the base peak of *m/z*= 44 due to the presence of extra amine-attached-methyl group. Other *m/z* values, i.e. *m/z*=77 and *m/z*=105 will show in case of methcathinone similarly to the ones appeared in case of cathinone (Figure 58)

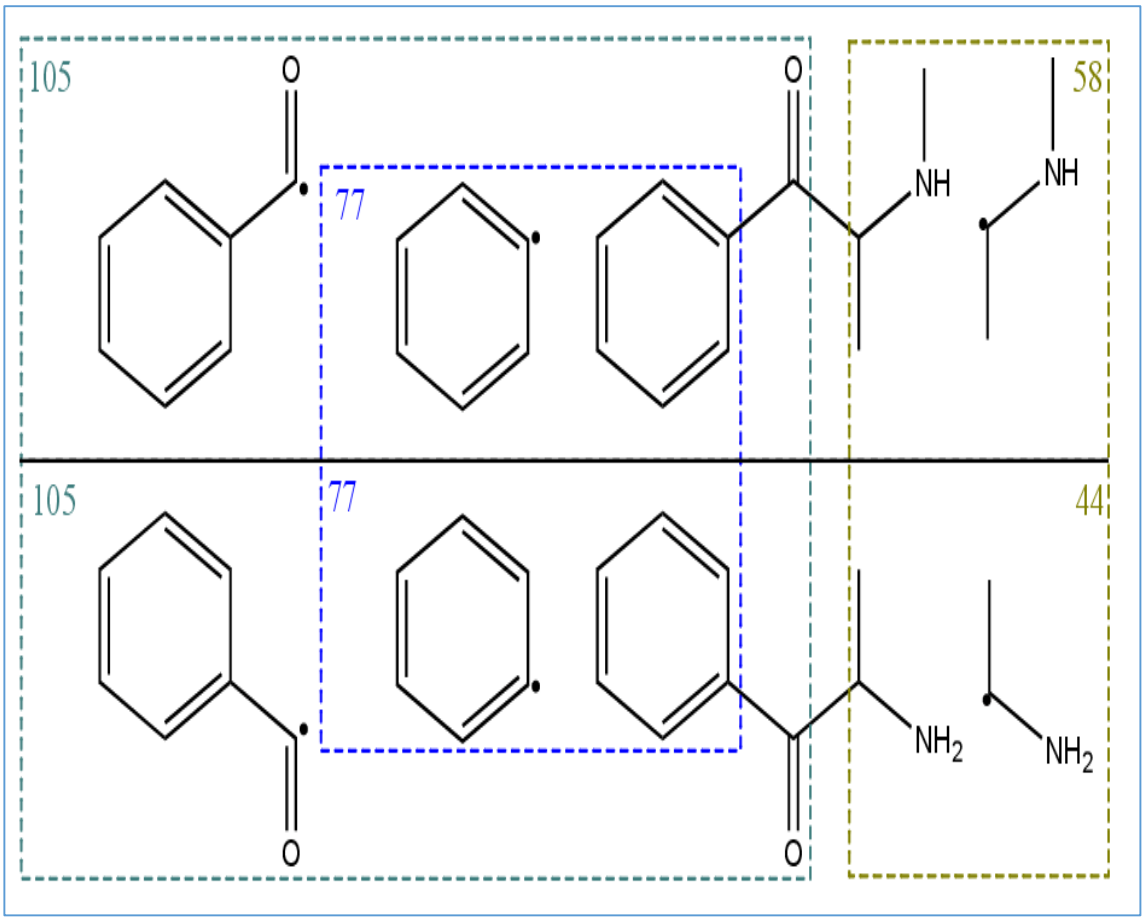


Figure 58: Expected fragmentation pattern of methcathinone (upper) in compare with fragmentation pattern of cathinone (lower).

Same as was noticed in case of mephedrone, two adjacent peaks were detected, the first peak retention time was 6.79 and the second peak retention time was at 6.90 minutes (Figure 59).

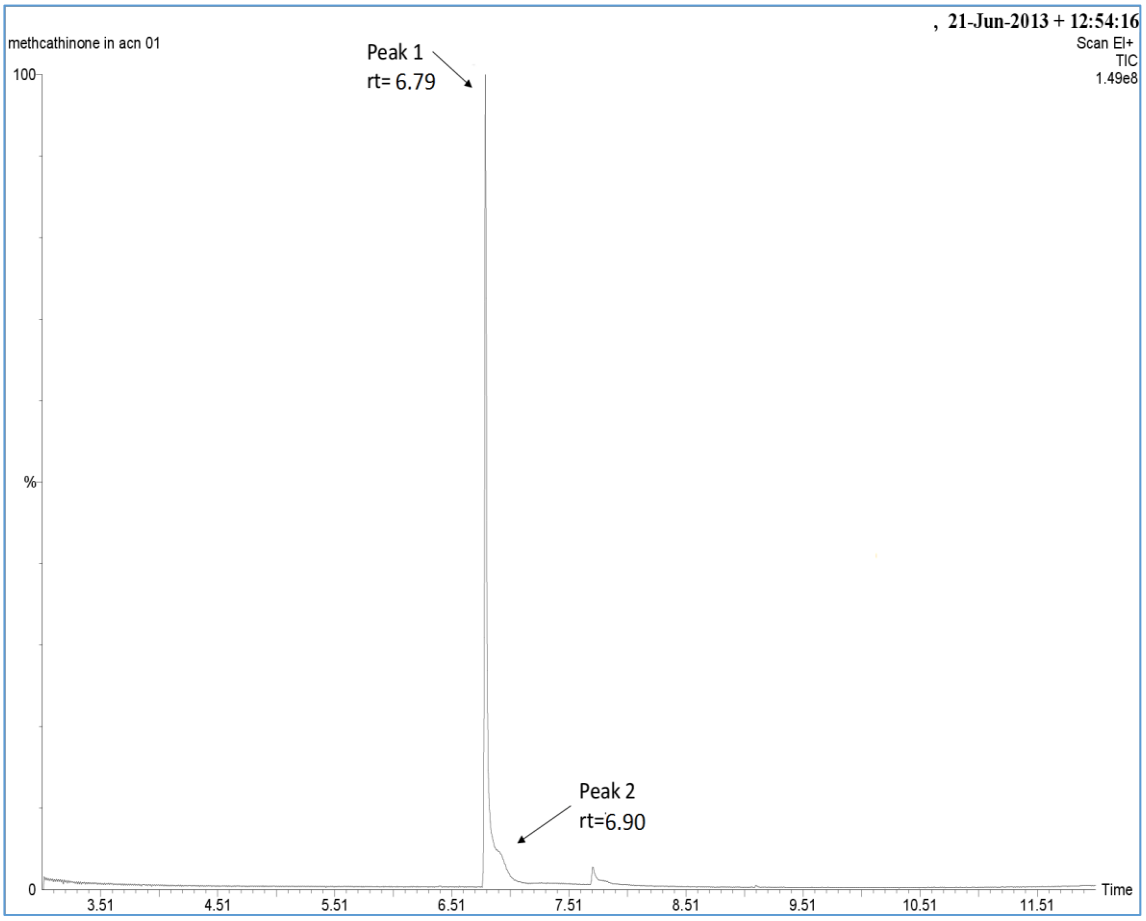


Figure 59: Chromatogram produced after injection of methcathinone onto GC-MS system

As expected from the proposed fragmentation pattern and in compare with the mass spectra of cathinone, mass spectra of the first peak showed the molecular ion with *m/z* 163 in addition to *m/z* values of 148, 133, 119, 91 and 58. The base peak was *m/z* 58 that corresponds to the peripheral ethyl-methyl-amine group, a product of α-cleavage (Figure 60)

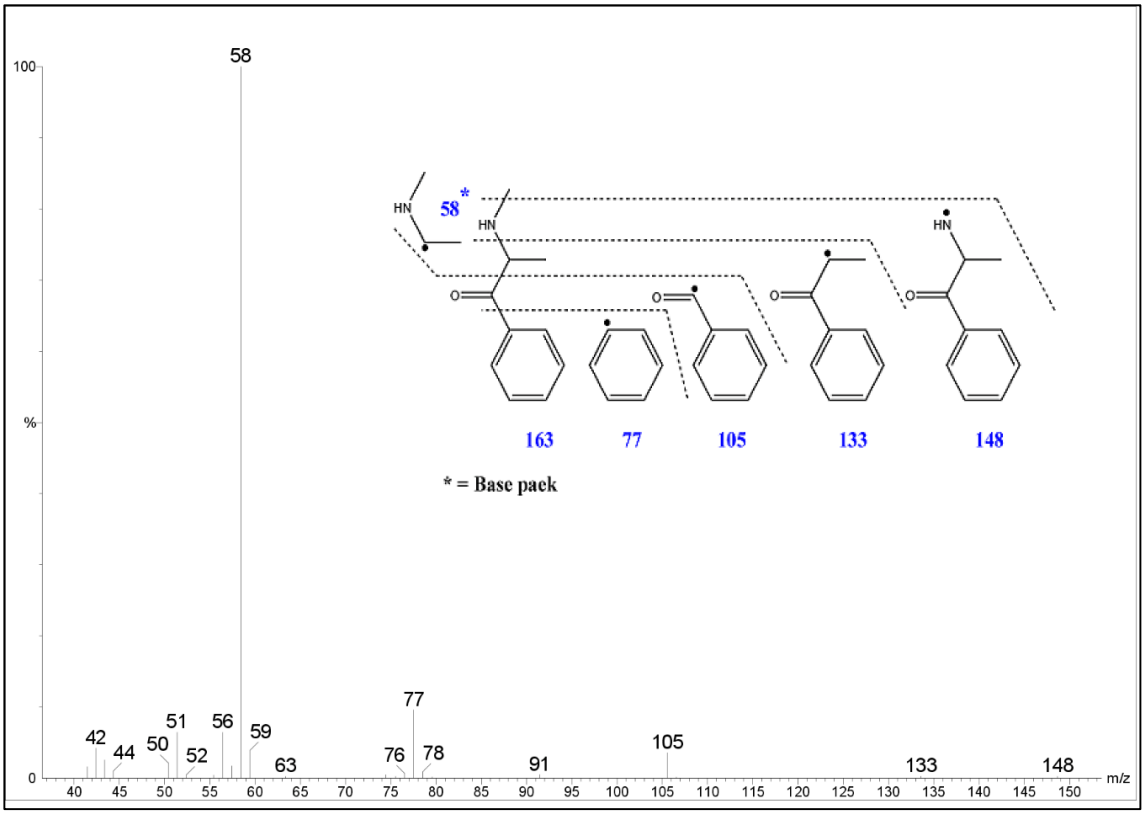


Figure 60: Mass spectrum of the first detected peak after injection of methcathinone onto GC-MS system and the proposed fragmentation pattern

Checking the relative abundance of each detected *m/z* value and its corresponding isotope, they were relatively close to the expected natural occurrence for the *m/z* values of 58, 77, and 105 (Table 17).

Table 17: Natural occurrence and relative abundance of *m/z* value and its corresponding isotope for methcathinone.

***m/z* vs isotope**	**Natural Occurrence**	**Relative Abundance**
58 vs 59	**96.8% vs 3.2%**	**96.5% vs 3.5%**
77 vs 78	**93.7% vs 6.3%**	**92.8% vs 7.2%**
105 vs 106	**92.7% vs 7.3%**	**92.6% vs 7.4%**

Again, and same as noticed for mephedrone, the second detected peak for methcathinone showed comparable mass spectrum, mainly with the appearance of the base peak as less two masses in compare to the one detected for the first peak. Same explanations may be suggested: impurity, by-product, and methcathinone related molecule during synthesis, effect of pyrolysis processes upon injection or most likely formation of double N-C bond producing ketamine molecules. The repetition

of the same phenomenon – the appearance of the less two mass units in different occasion for different drugs - is more likely supportive for the assumption that it is due to 'analytical conditions 'or sample preparation steps leading to the same pattern, i.e. pyrolysis or the formation of the double bond producing ketoiminie molecules (Figure 61).

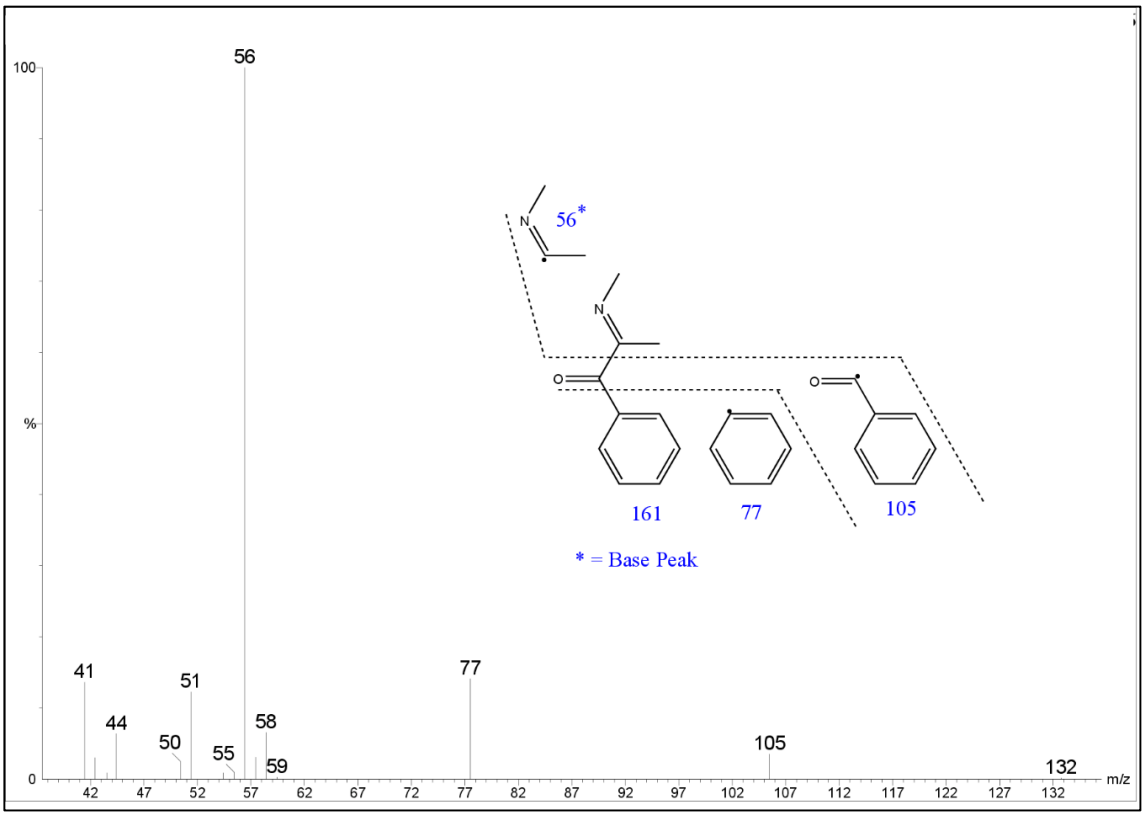


Figure 61: Mass spectrum of the second detected peak after injection of methcathinone onto GC-MS system and the proposed fragmentation pattern

More through studies are needed for the determination of this phenomenon, which may include study of other similar dugs – cathinones and synthetic cathinones. The importance of future such study is that these drugs are possible by-products or drug related chemicals that are underestimated by the scientific community, as those 'proposed chemicals' are potentially having psychoactive effects. The clinical and toxic effects of any drug may be related to other impurities or adulterants that may cause or augment toxic effects.

The derivatization of methcathinone produced a chromatogram with only one identifiable peak for the derivatized drug. Mass spectrum of this peak showed the base peak of *m/z*=130, the corresponding base peak of *m/z*=58 for the underivatized molecule, in addition to another peak of *m/z*= 220

confirming successful derivatization of methcathinone through replacement the active hydrogen of the amine group by the trimethylsilyl functional group (Figure 62). No other identifiable peaks were detected that were suggestive or confirmative of the derivatization of the enol form of methcathinone in a similar way to what was observed for mephedrone.

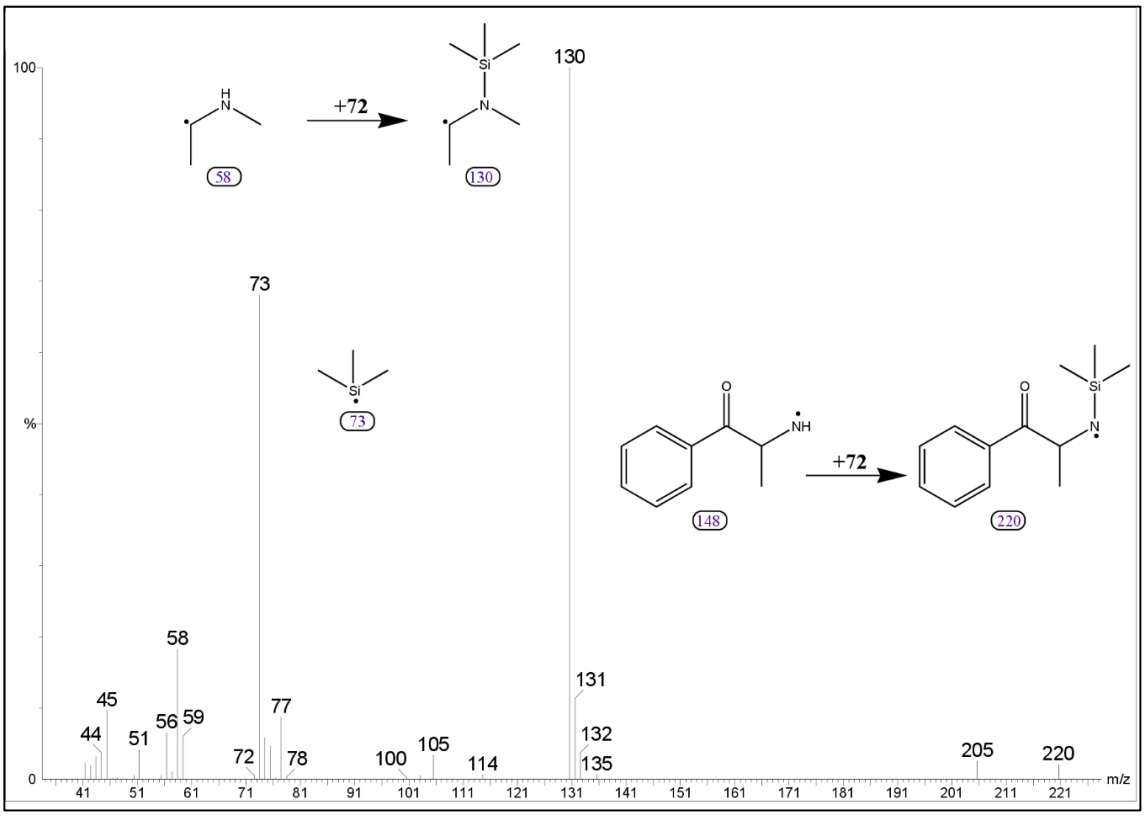


Figure 62: Mass spectrum of the second detected peak after injection of derivatized methcathinone onto GC-MS system

4.3.1.3. 4-fluoromethamphetamine (4-FMA)

4 –Fluromethamphetamine (4-FMA) is structurally similar to amphetamine, both having an (± alkyl) amine group and peripheral phenyl moiety. The fragmentation pattern of 4-FMA is expected to follow the same fragmentation pattern of amphetamine, where it will show base peak of *m/z*=58 in comparison to the base peak of *m/z*= 44 due to the presence of extra amine-attached-methyl group. Due to the presence of fluorine attached to the phenyl moiety in 4-fluoromethamphetamine, secondary peaks of *m/z*=77 and *m/z*= 91 in case of amphetamine will be shown as mass peaks *m/z*= 95 and *m/z*=109 respectively (Figure 63).

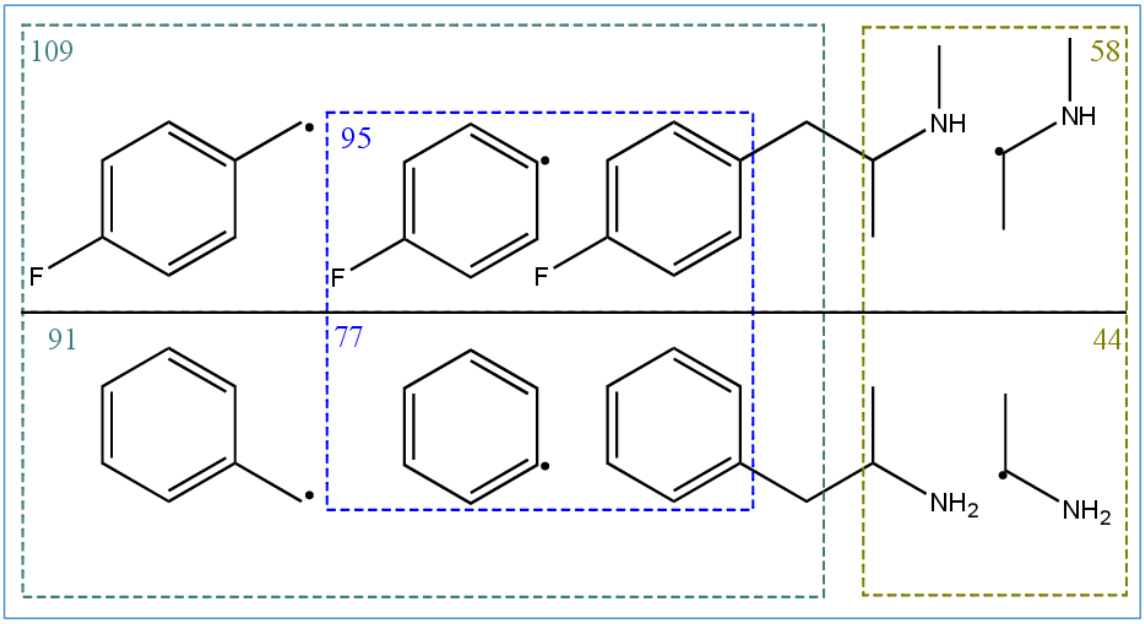
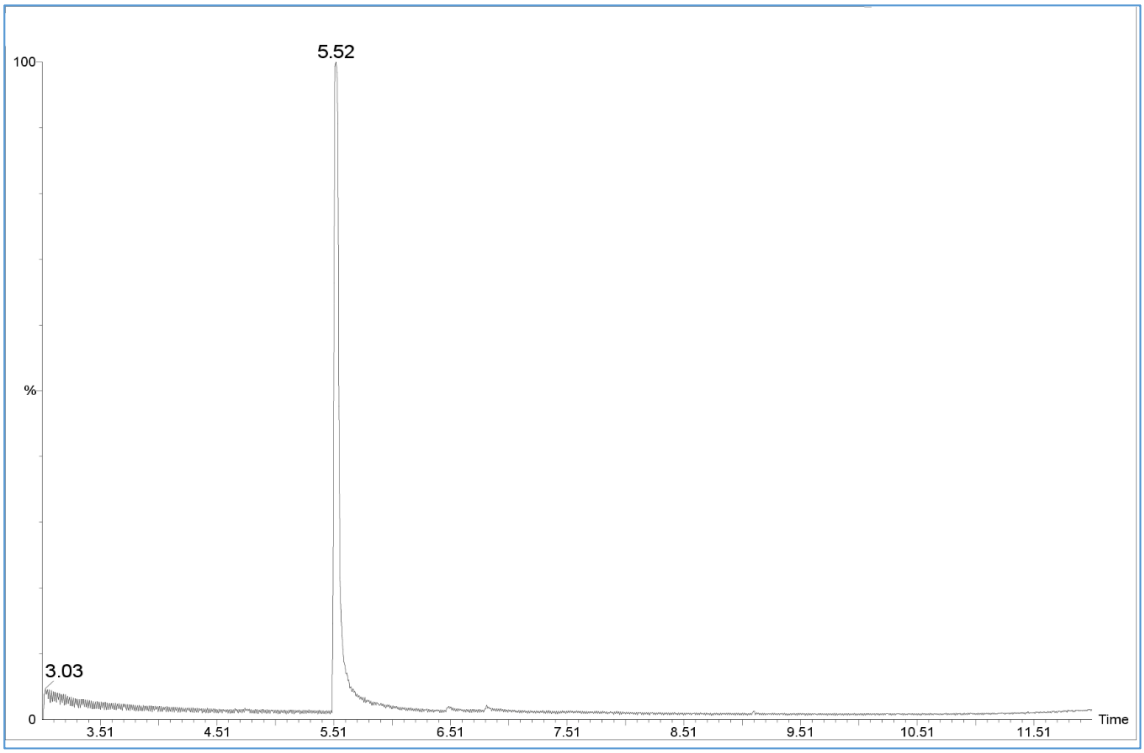


Figure 63: Expected fragmentation pattern of 4-FMA (upper) in compare with fragmentation pattern of amphetamine (lower).

Standard of the 4-FMA were injected onto the GC-MS system, and resultant mass spectra was compared with the proposed fragmentation pattern. One peak was detected with retention time of 5.52 minutes. As expected from the proposed fragmentation pattern and in comparison with the mass spectra of amphetamine, in addition to the base peak of *m/z* 58 that corresponds to the peripheral ethyl-methyl-amine group, a product of α-cleavage, mass spectra showed *m/z* values of 152, 137, and 109.



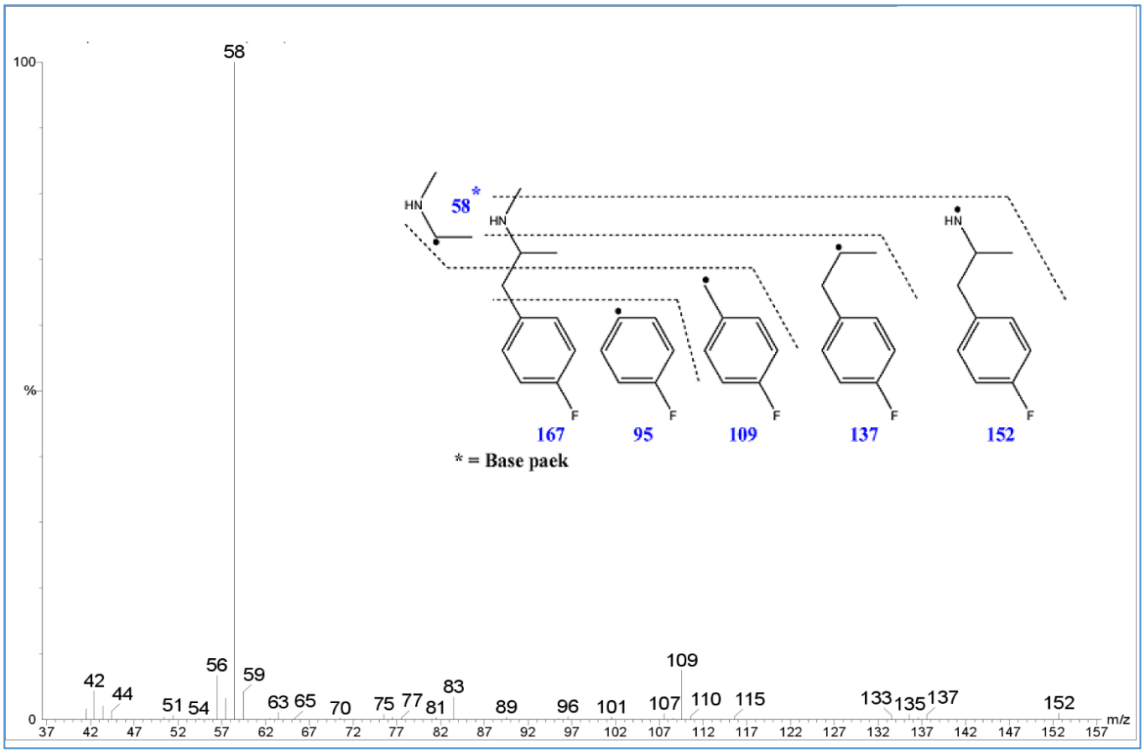


Figure 64: Chromatogram and Mass spectrum of the detected peak after injection of 4-FMA onto GC-MS system and the proposed fragmentation pattern

Another indicative tool, similar to what was applied previously, is to check that the relative abundance of each detected *m/z* value, against its corresponding isotope values of 58 and 109, were relatively close to the detected relative abundance. The corresponding isotope value of 95 was not detected (Table 18).

Table 18: Natural occurrence and relative abundance of *m/z* value and its corresponding isotope for 4-FMA.

m/z vs isotope	Natural Occurrence	Relative Abundance
58 vs 59	96.8% vs 3.2%	96.2% vs 3.8%
109 vs 110	92.7% vs 7.3%	93.1% vs 6.9%

The derivatization of 4-FMA produced a chromatogram with only one identifiable peak for the derivatized drug. Mass spectrum of this peak showed the base peak of *m/z*=130, the corresponding base peak of *m/z*=58 of the underivatized molecule (Figure 65).

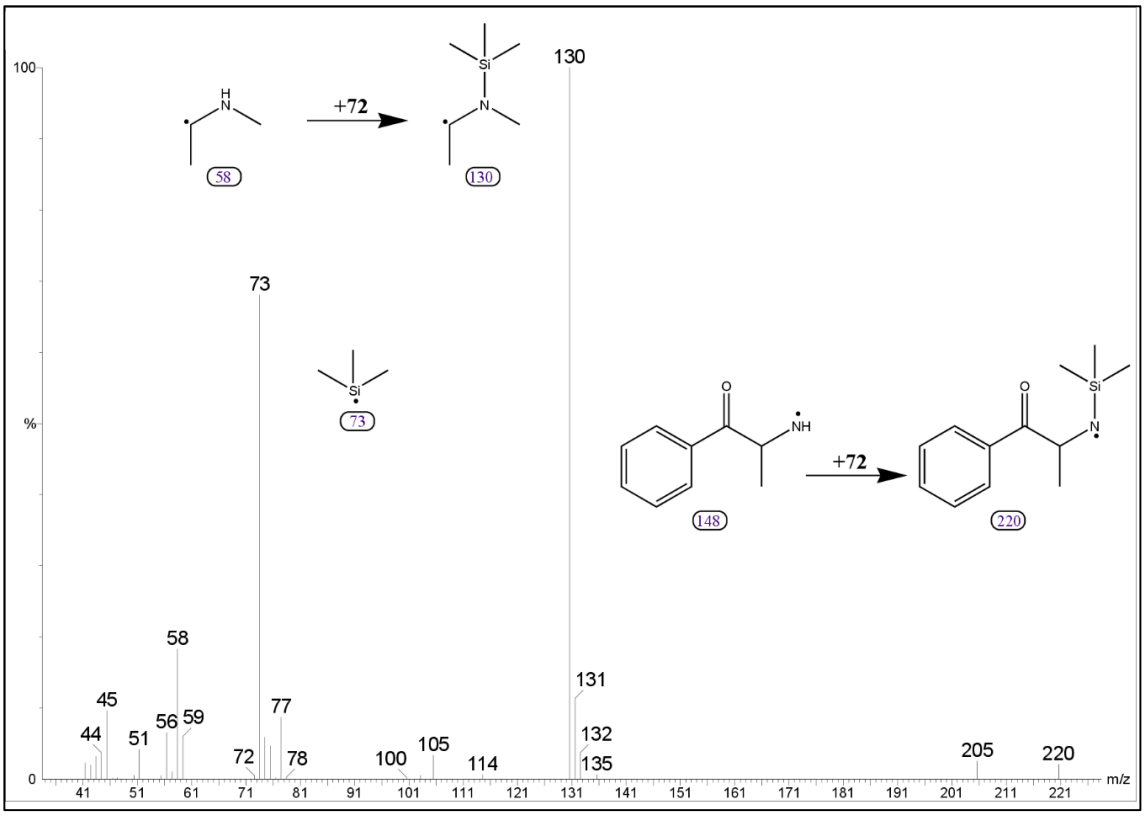


Figure 65: Mass spectrum of the detected peak after injection of derivatized 4-FMA onto GC-MS system

4.3.1.4. Methoxetamine (MXE)

Methoxetamine, as discussed earlier, is structurally similar to Ketamine and belongs to the arylcyclohexylamine family, and so expected both to have similar fragmentation pattern. Fragmentation of ketamine and its metabolite was studied utilizing LC/MS, and the authors proposed a pattern where the ketamine shows neutral loss of H_2O and a loss of ethylamine followed by loss of carboxyl or H_2O (Wang et al., 2005). Another recent study for the characterization of methoxetamine, with comparison to ketamine utilizing different instrumentation, including mass spectrometry, proposed a comprehensive fragmentation pathway for methoxetamine (Hays et al., 2012). The latter study presented both spectra of ketamine and methoxetamine, and suggested that the major fragmentation of methoxetamine would be similar to ketamine due to the similarity of structure. We expected this similarity in pattern earlier in our work, and suggested this similarity in structure would produce a similar fragmentation pattern as well as to be metabolized in a relatively similar pattern.

Standard of MXE were injected onto the GC-MS system, and resultant mass spectra was compared with the proposed fragmentation pattern. One signal was detected with retention time of 10.40

minutes. The mass spectrum of the detected peak for MXE produced *m/z*=190 as the base peak and the following *m/z* values that were suggestive to be the fragments of MXE: 219 and 176 (Figure 66).

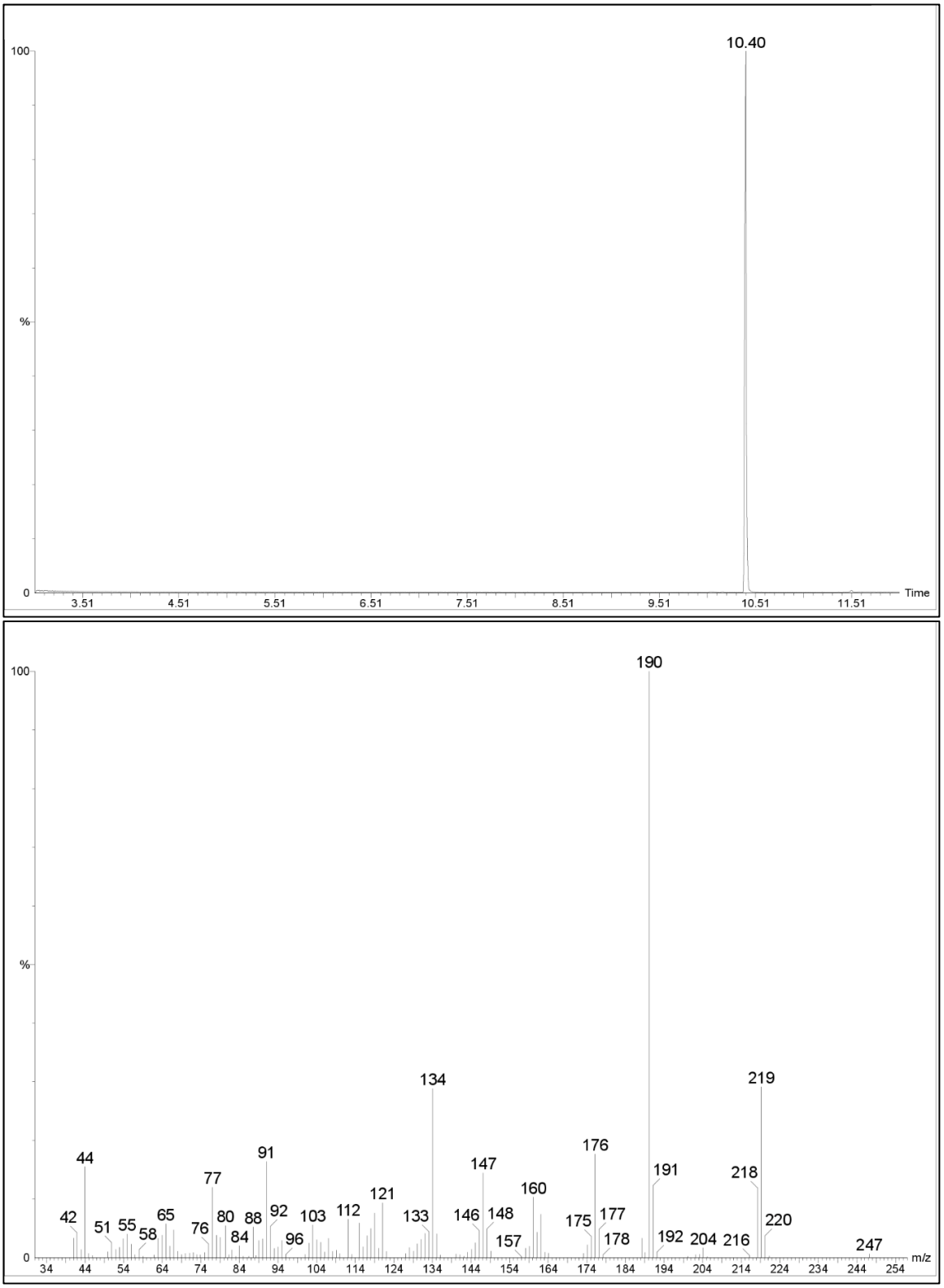


Figure 66: Chromatogram produced and Mass spectrum of the detected peak after injection of derivatized MXE onto GC-MS system

As proposed earlier by Hays et al, MXE goes into α-cleavage and loss of the ketone part as CO and following ring closure (Hays et al., 2012). In this part of the study, fragment ions at *m/z* 134, 147, 176,177, 190, 218, and 276 were also characteristic for MXE. As shown in spectrum 2, ring degradation by further loss of carbon atoms led to fragment ion at *m/z* 134 and 121. Meyer et al studied later metabolism and toxicological detectability of MXE using GC-MS and similar results were observed (Meyer et al., 2013). Combining the previous and later studies, with the detected mass spectrum of MXE, a possible fragmentation pathway of MXE can be proposed (Figure 67).

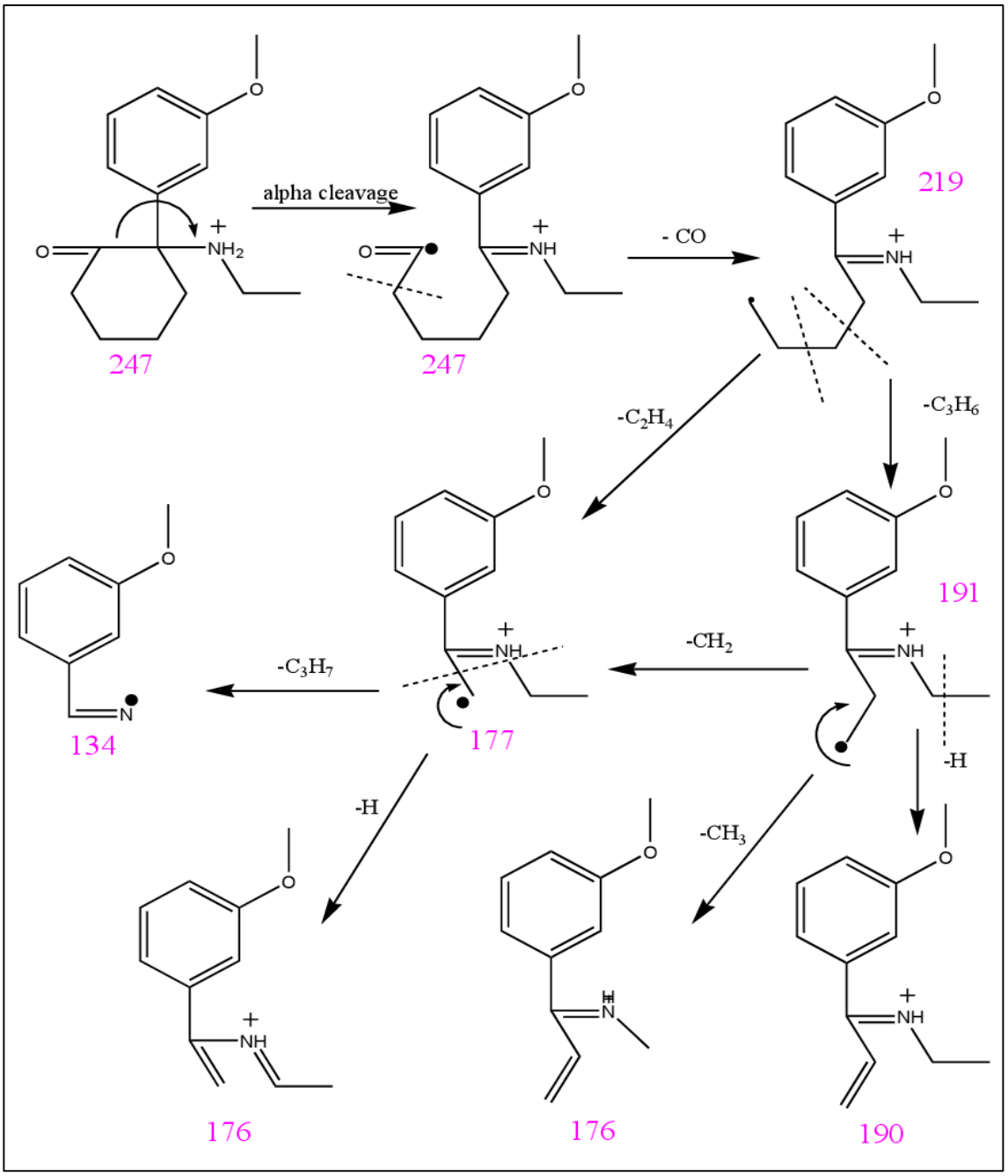


Figure 67: Predominant fragments structure and proposed fragmentation pathway of MXE after injection onto GC-MS

The derivatization of MXE produced a chromatogram with only one identifiable peak for the derivatized drug. Analysis of the detected *m/z* values was confirmative for successful derivatization by replacement of active hydrogen of amine by trimethylsilyl group. Mass spectra showed characteristic *m/z* values of 319, 304 and 73 (Figure 68)

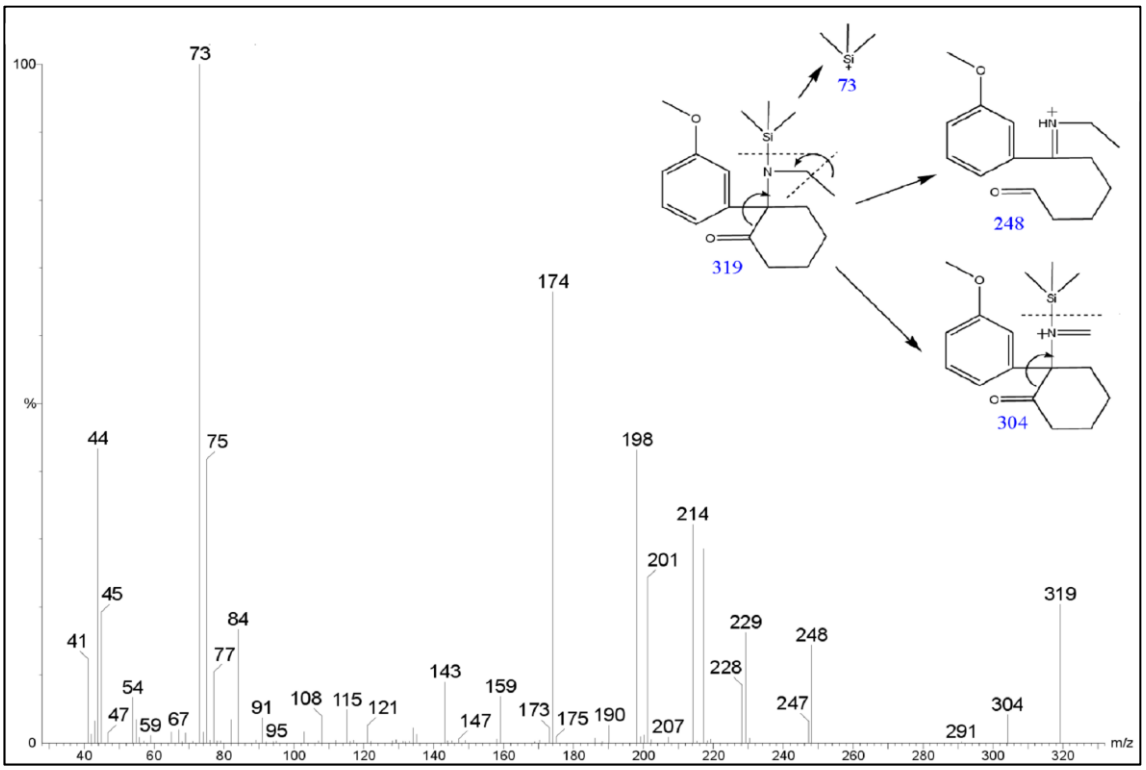
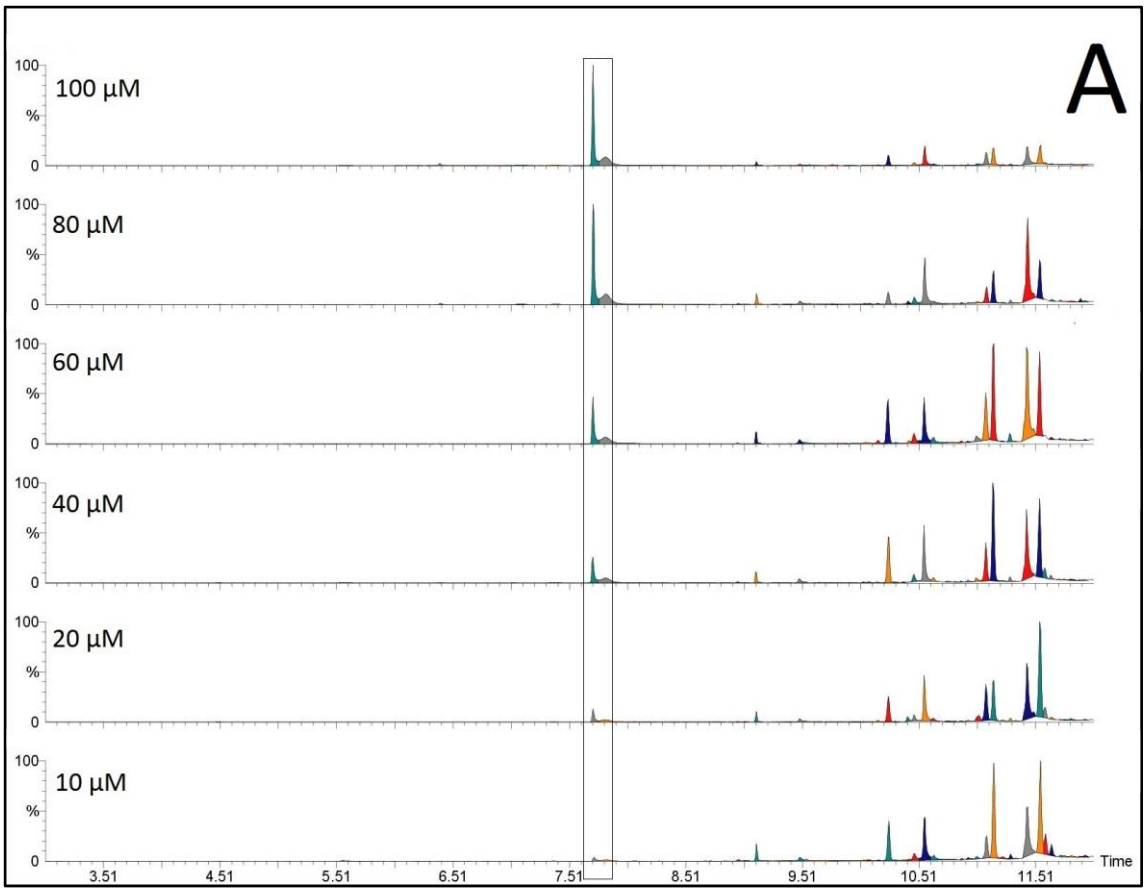


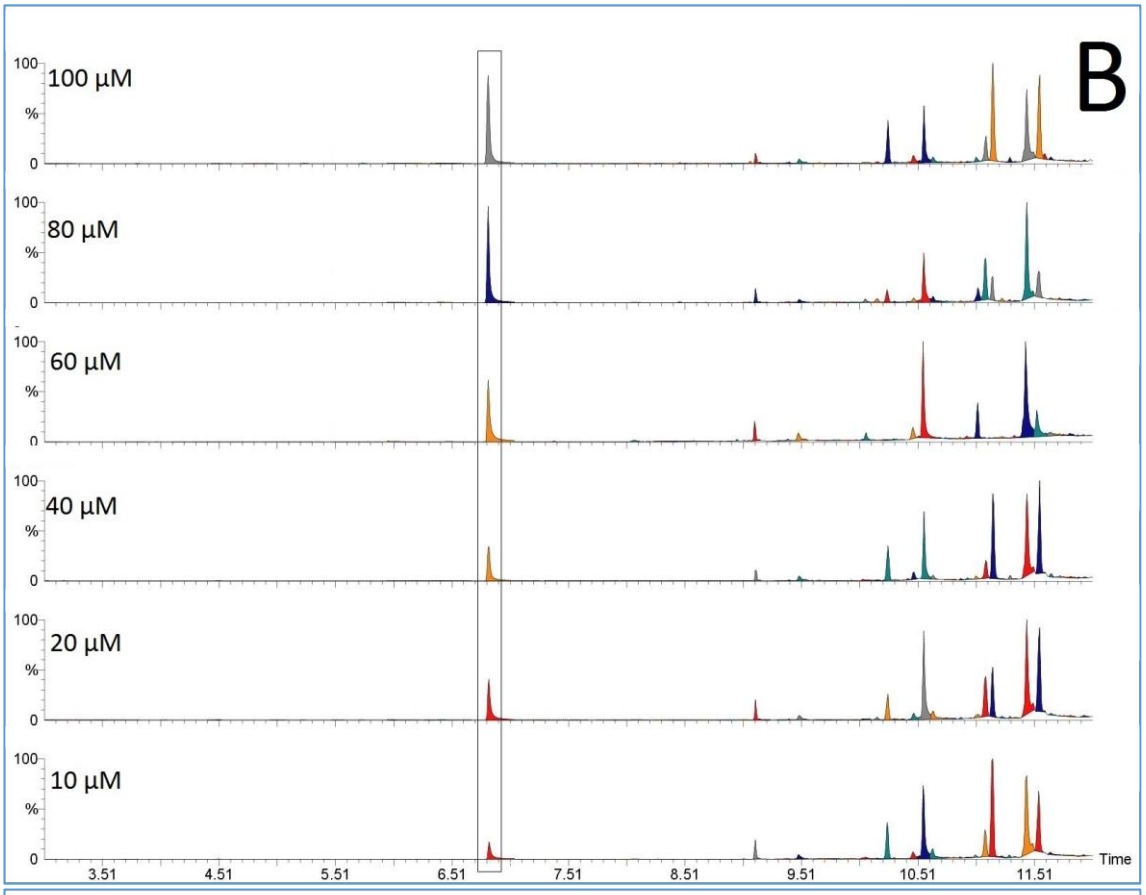
Figure 68: Mass spectrum of the detected peak after injection of derivatized 4-FMA onto GC-MS system

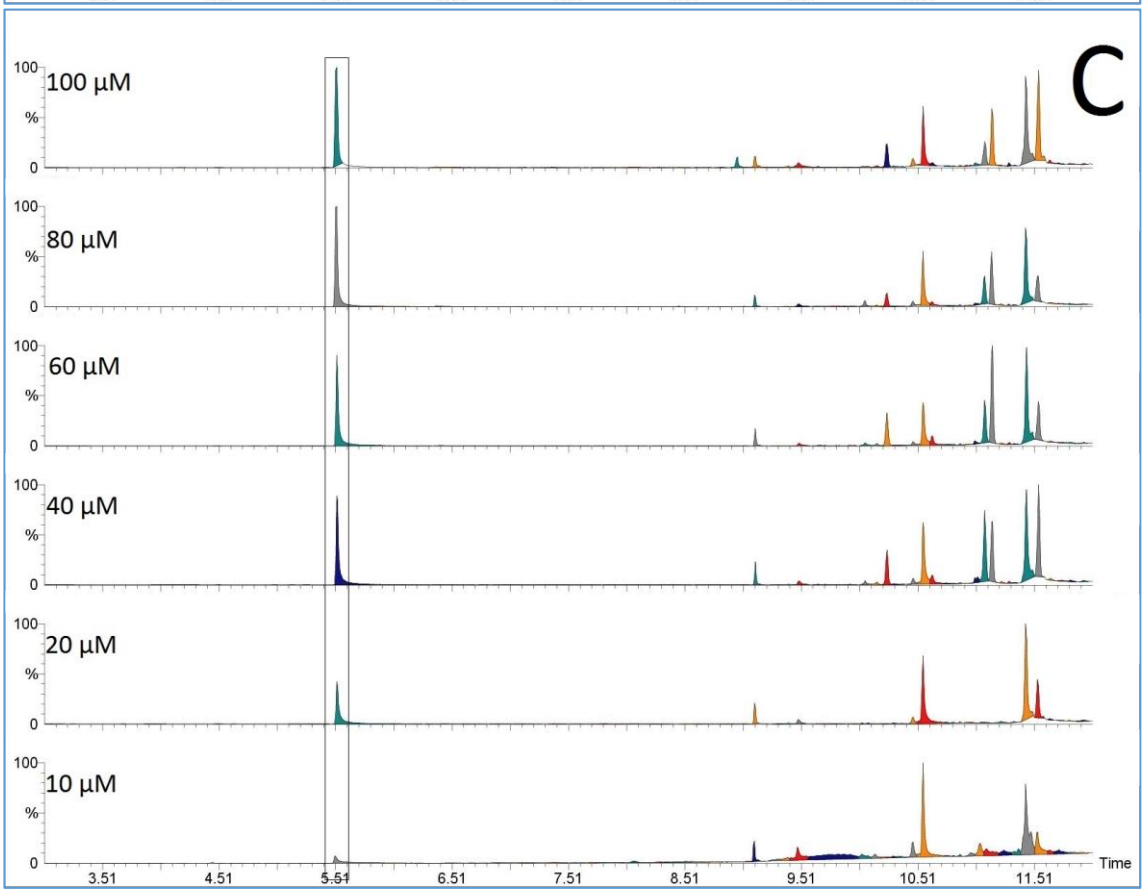
4.3.2. *In vitro* metabolism studies of selected NPS using HepaRG™ followed by analysis utilizing GC-MS

Following the analysis of the selected NPS utilizing GC-MS (section 4.3.1), HepaRG™ Cells were used as *in vitro* model for the study of the metabolism of the selected NPS (section 3.3.3.5). Each drug was studied at different concentrations (10, 20, 40, 60, 80 and 100 µM), and each concentration was studied at the following specified times (0, 90 minutes and 24 hrs). The chromatograms obtained in the experiment were studied for the presence of the parent drug and new peaks when compared to blank samples. Retention times and fragmentation patterns for each drug were used to confirm drug identity.

Parent drugs were detected in all samples and the identity of the drug was confirmed using retention times and mass spectra (see section 4.3.1.). For different concentrations of each drug used, peak areas were proportional to the drug concentrations, where higher concentration was reflected by larger peak areas (Figure 69).




B
100 µM
80 µM
60 µM
40 µM
20 µM
10 µM
100
%
0
3.51
4.51
5.51
6.51
7.51
8.51
9.51
10.51
11.51
Time


C
100 µM
80 µM
60 µM
40 µM
20 µM
10 µM
100
%
0
3.51
4.51
5.51
6.51
7.51
8.51
9.51
10.51
11.51
Time

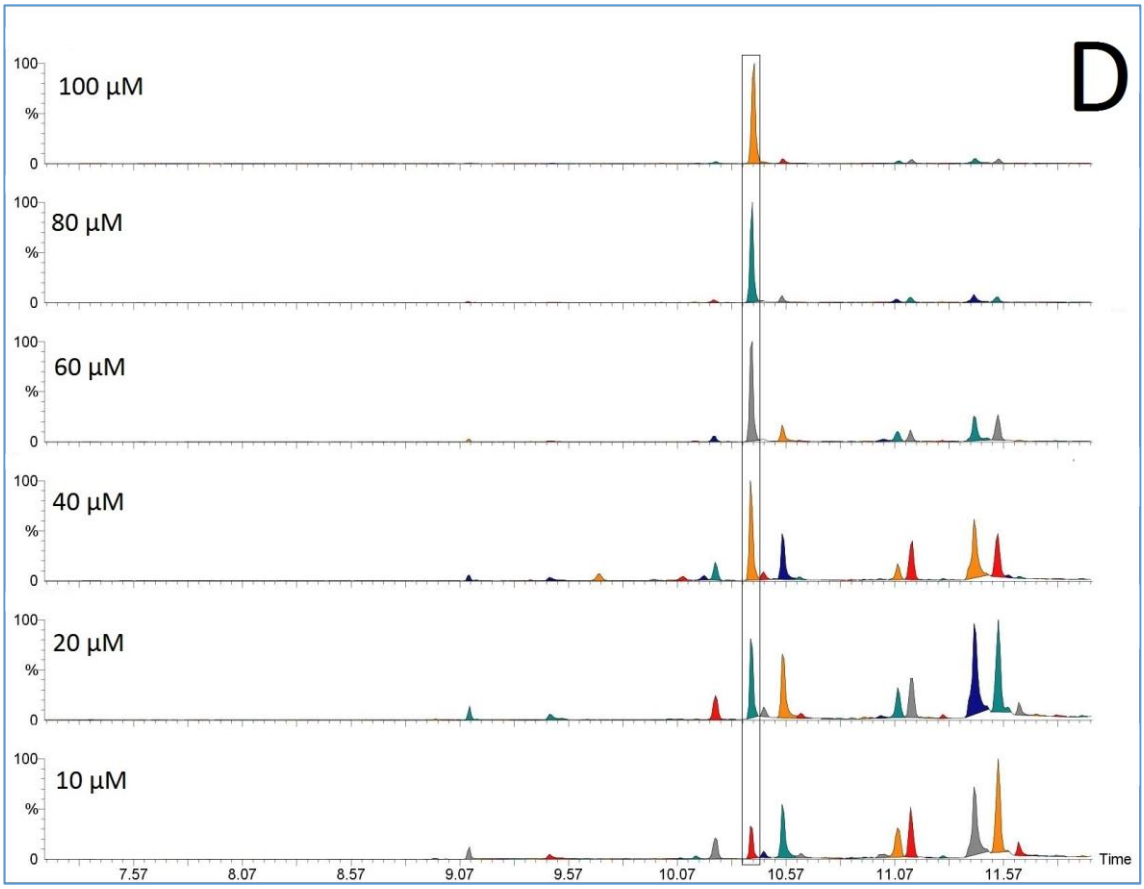
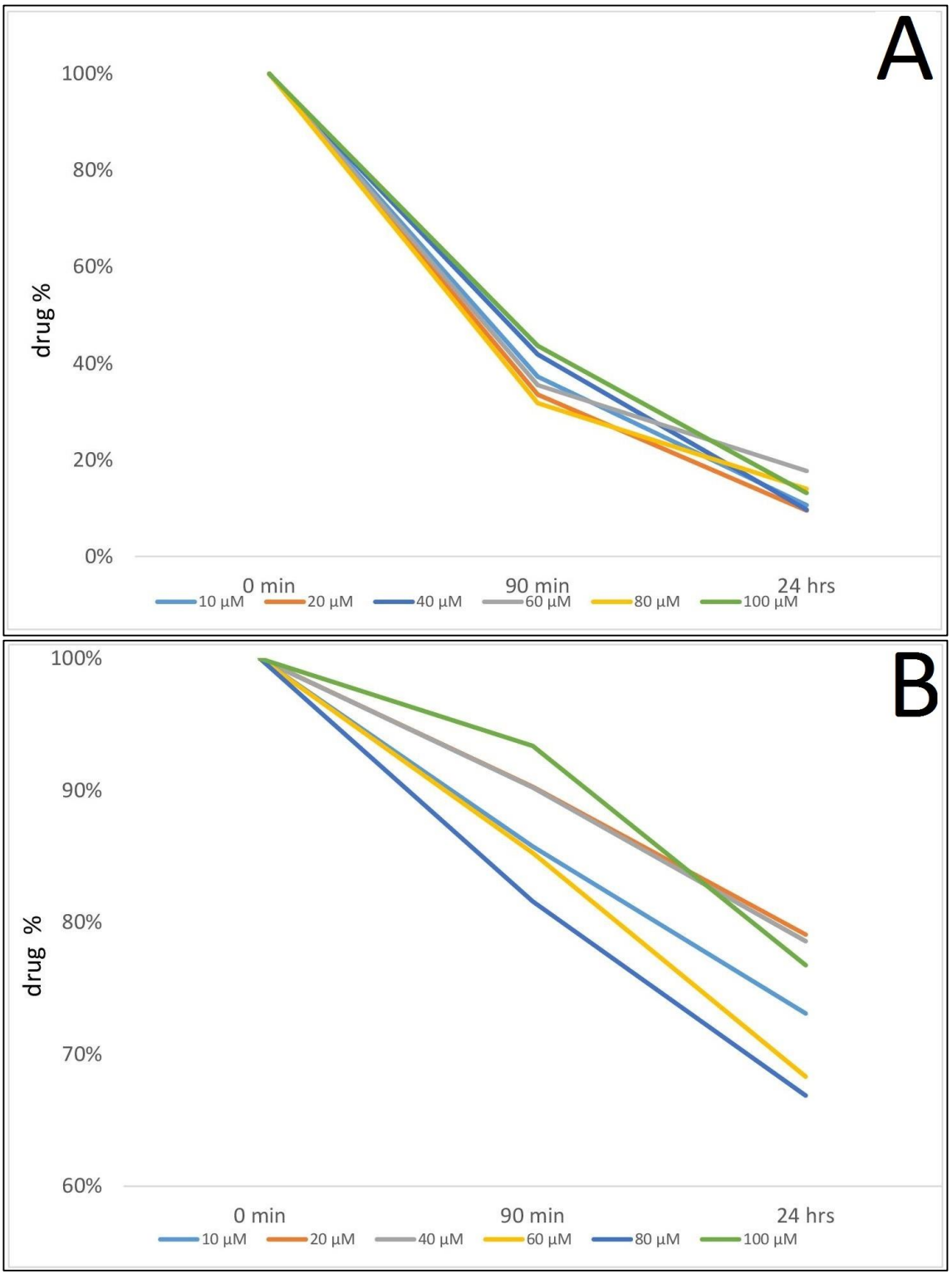


Figure 69: chromatograms of selected NPS of different concentration at zero time after incubation with HepaRG™.
Squared peaks are of the parent drug, where: A: mephedrone, B: methcathinone, C: 4-fluoromethaphetamine and D: methoxetamine

Peak areas were plotted against concentration for linearity, and for all selected NPS showed to be linear with $R^2 > 0.96$ (Figure A.74, p. 186). The data collected were plotted as percentage of drug concentration in relation to the primary drug concentration at zero time, reflected by average peak area (n=2), and the resultant relative concentrations were plotted against time. Mephedrone, methcathinone and 4-fluoromethamphetamine showed comparable trend of drugs concentration over time of incubation with HepaRG™ cell line. These drugs showed continuous decrease in drugs concentration over time, while methoxetamine showed a sharp decrease in drug concentration followed by an increase.

For mephedrone, the drug concentrations relative to the primary drug concentration dropped to an average of 37% (32-44%) after 90 minutes of incubation with HepaRG™, and then there was another decrease down to an average of 12% (9-18%) after 24 hrs. In a comparable trend, methcathinone and

4-fluoromrthamphetamine concentrations dropped to averages of 88% (82-93%) and 85% (80-93%), respectively, after 90 minutes of incubation with HepaRG™. This decrease was followed by another decrease down to 74% (67-79%) and 76% (70-82%) for methcathinone and 4-fluoromethamphetamine, respectively (Figure 70).



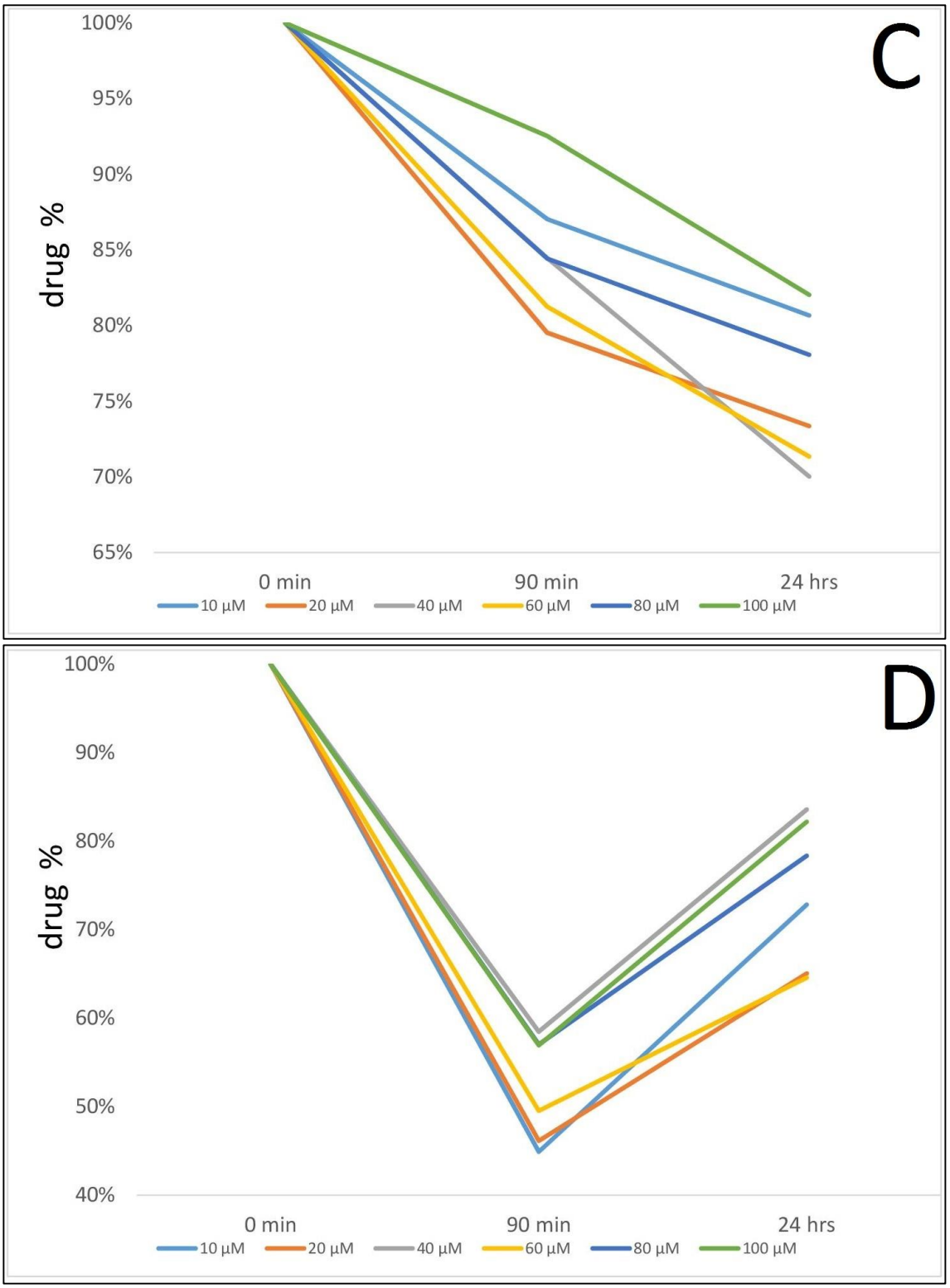


Figure 70: relative drug concentrations of selected NPS to the primary drug concentration after 90 minutes and 24 hours of incubation with HepaRG™.
A: mephedrone, B: methcathinone, C: 4-fluoromethamphetamine and D: methoxetamine.

The structural similarity of mephedrone, methcathinone and 4-fluoromethamphetmaine may suggest similar physicochemical properties, which may explain why these drugs behave similarly in

biological systems in terms of, for example, transcellular transport, protein affinity and metabolism. Protein binding is one major component that affect drug's efficacy, as it is only the free unbound fraction of the drug that is available to be transported to the intracellular space for further biological action, e.g. metabolism. In comparison, methoxetamine showed different concentrations over time, which could be explained by the different structural backbone in comparison to the other selected NPS. However, the increase in methoxetamine concentration after 24 hours of incubation after the sharp decrease after 90 minutes could be due to cell death and lysis, which caused the intracellular content of methoxetamine to pool into the extracellular space. This general trend of lower available extracellular drug concentration is a strong evidence that drugs are being used or consumed by the cells, most likely through metabolism.

By studying the chromatograms of the selected NPS, at different concentrations and different incubation times, for new peaks, in comparison to blank controls, it was possible to detect one new peak in mephedrone samples and one new peak in methcathinone samples. In both cases the new peaks appeared earlier before the parent drug. It is expected that any potential metabolites of the drug would be more polar than the parent drug, and would appear earlier in the chromatogram when using reversed mode chromatography, the one used through this thesis work.

Studying the chromatograms of mephedrone after incubation with HepaRG™, a new peak was detected, before the parent drug, at 6.39 minutes. The mass spectrum of these new peak showed a comparable mass spectrum to that of mephedrone. The mass spectrum of this peak showed both *m/z* values of 119 and 91. These two *m/z* values are the common masses appearing in the mass spectrum of mephedrone together with the base peak with *m/z* value of 58 (see Figure 53). This pattern may suggest that the new peak is related to the parent drug with structural change affecting the amine group (Figure 71). In fact, N-demethylation is one of the most reported pathways for metabolism of mephedrone and structurally similar drugs, which may suggest the detected peak is the product of N-demethylation. However, it was not possible to detect the expected *m/z* value of 44, a product of the suggested N-demethylation pathway.

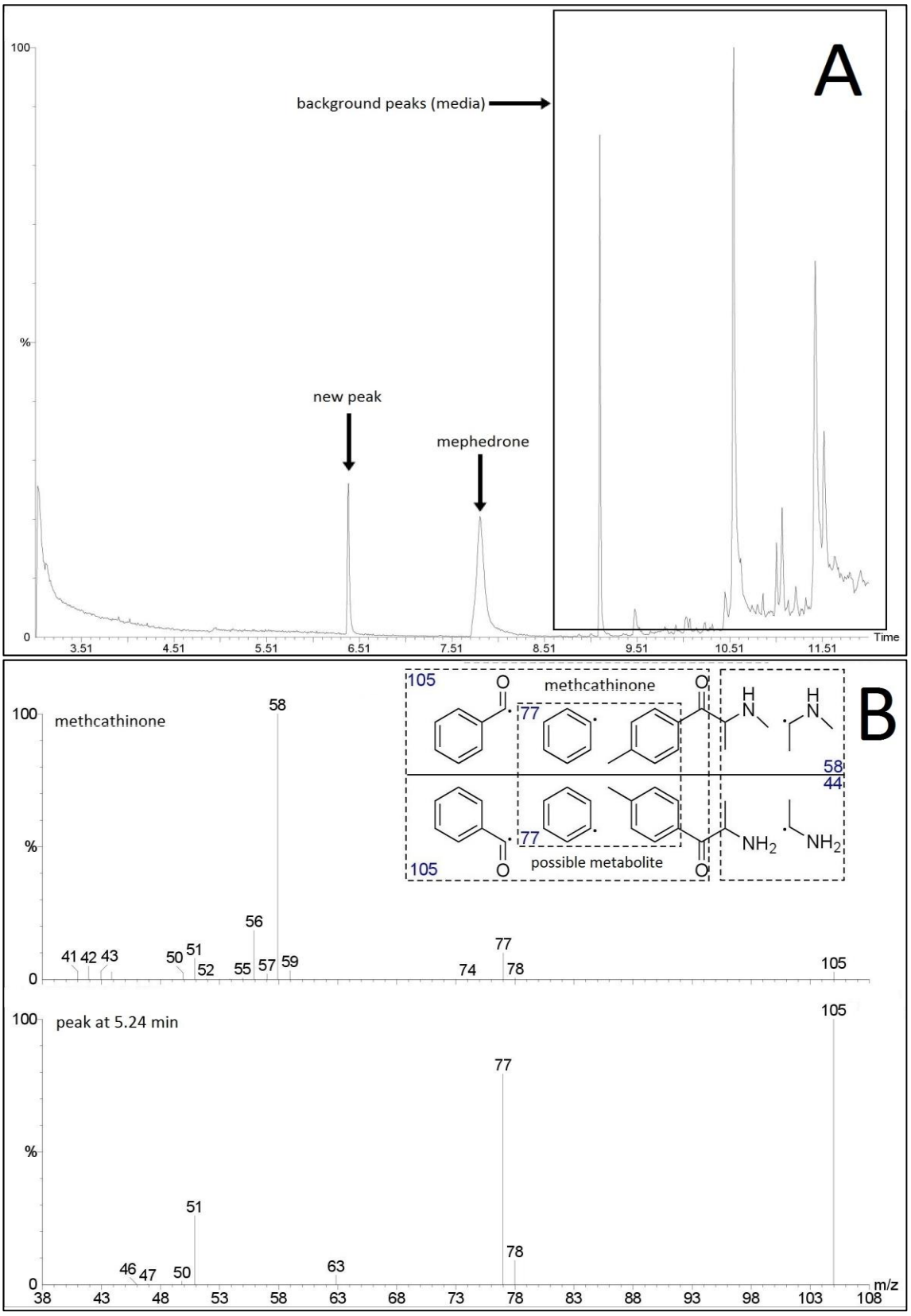
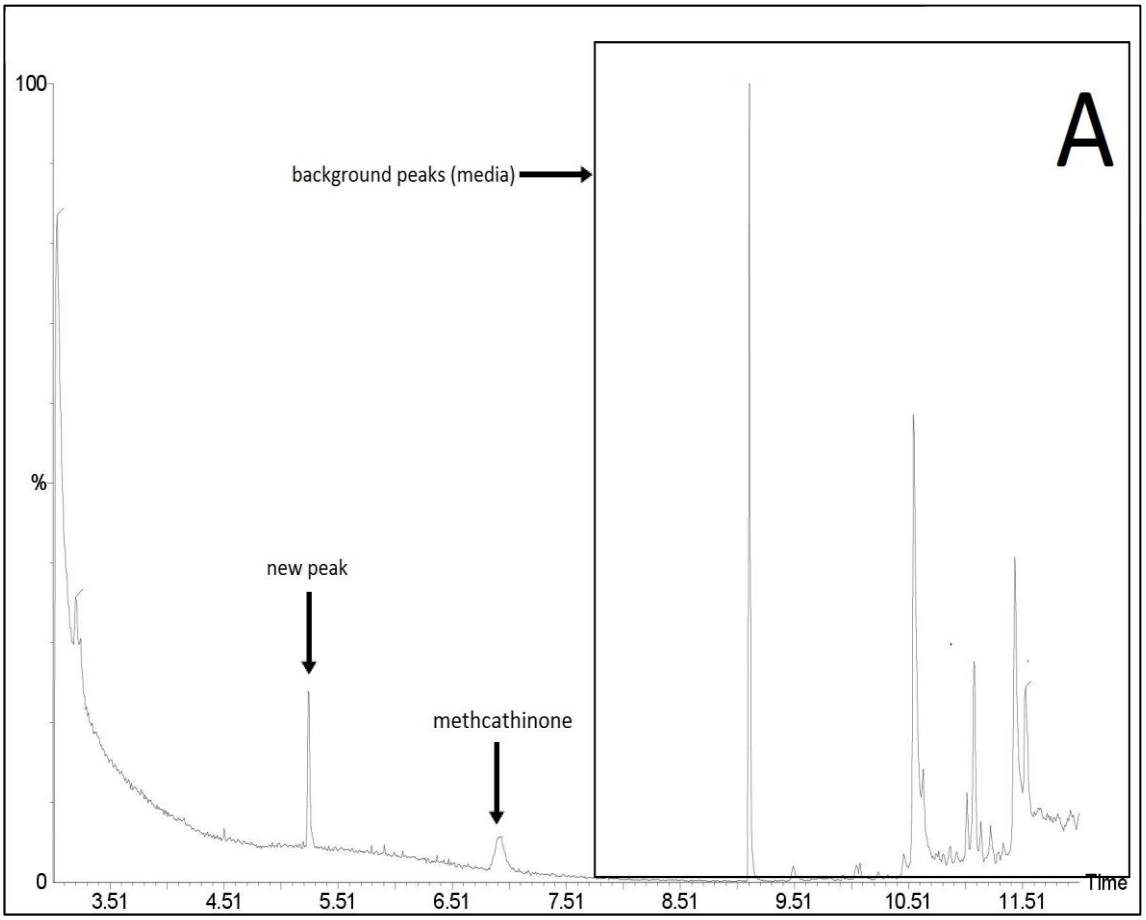


Figure 71: sample chromatogram (A) and mass spectrum of newly appearing peak of samples of mephedrone after incubation with HepaRG™.

In a similar approach, chromatograms for methcathinone showed a new peak at 5.24 minutes when compared to blank controls. The mass spectrum of this peak was also comparable to that of methcathinone. The mass spectrum showed both *m/z* values of 105 and 77. These two *m/z* values are common masses appearing in mass spectrum of methcathinone together the base peak with *m/z* value of 58 (see Figure 60). This is a similar pattern to the one observed for mephedrone, suggesting this new peak to be related to methcathinone with structural change affecting the amine group (Figure 72). This may suggest that methcathinone, similar to mephedrone, underwent N-demethylation. The structural similarity between mephedrone and methcathinone, both being β-ketoamines, may explain the correlation observed in the analysis of mephedrone and methcathinone analysing samples of these drugs after incubation with HepaRG™.



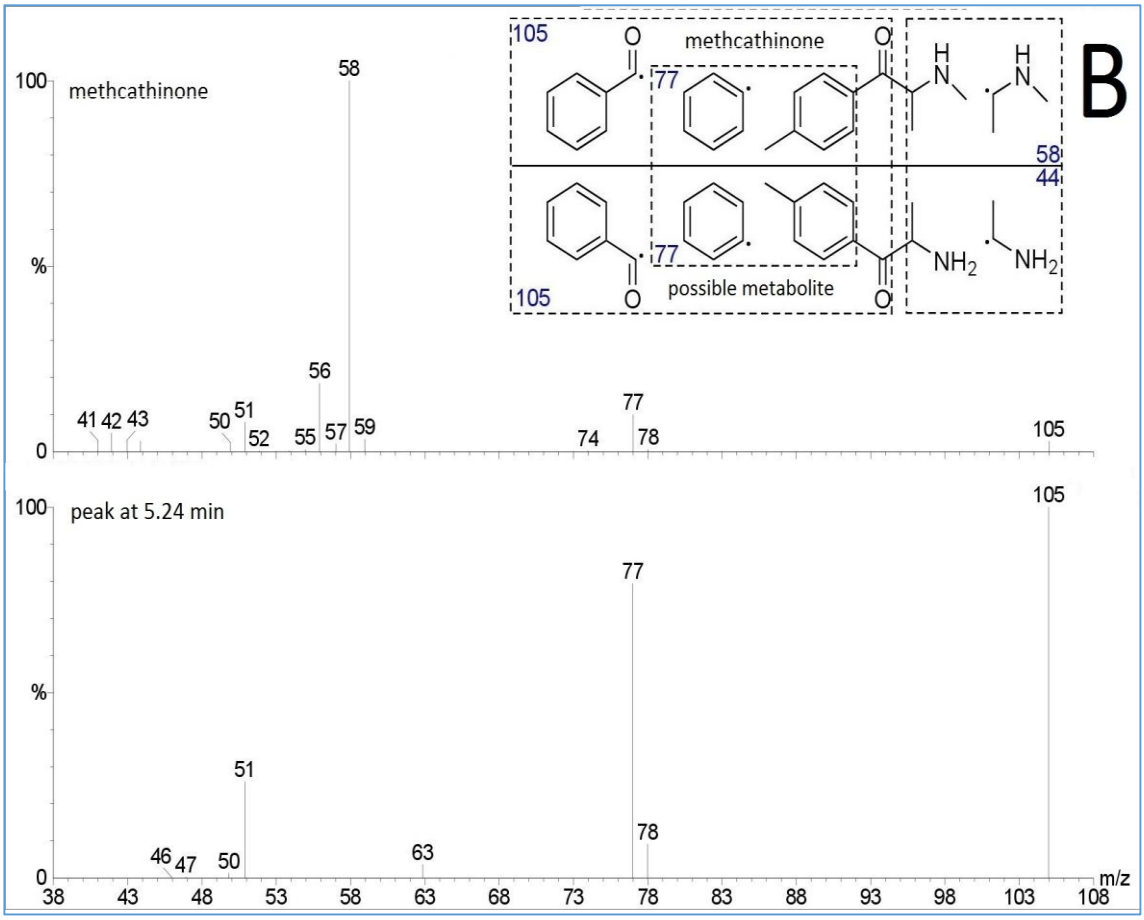


Figure 72: sample chromatogram (A) and mass spectrum of newly appearing peak of samples of mephedrone after incubation with HepaRG™.

In chromatograms obtained for 4-fluoromethamphetamine and methoxetamine after incubation with HepaRG™, no new peaks were detected.

In general, though these drugs were successfully analysed utilizing GCMS, any potentially produced metabolites are expected to be more polar, less volatile and less stable when analysed utilizing GCMS, which makes LCMS a better option for analysis of any potentially metabolites. However, derivatization of the samples is expected to increase volatility and stability of the compounds, which increases detectability of potentially produced metabolites utilizing GCMS.

All samples of drugs after incubation with HepaRG™ were derivatized using BSTFA, but it was not possible to detect new peaks that could be correlated to any potentially produced metabolites.

4.3.3. Cytotoxicity studies of selected NPSs using MTT Assay utilizing HepaRG™

Mephedrone (4MMC), methcathinone (MCAT), 4-fluoromethamphetamine (4-FMA) and methoxetamine (MXE) were the selected NPS for this part of the study. 4MMC, MCAT and 4FMA are amphetamine related drugs with currently no available data about their cytotoxic effects. However, amphetamines, cathinones and related drugs have been studied, both *in vitro* and *in vivo*, for their cytotoxic effects. Cytotoxicity caused by amphetamines mainly affects the liver, the organ most at risk in general for drug related toxicity, and specifically amphetamines (Da Silva et al., 2013a). No similar data are available for MXE, the ketamine analogue belonging to the Arylcyclohexylamine, or other drugs of the same class, for comparison.

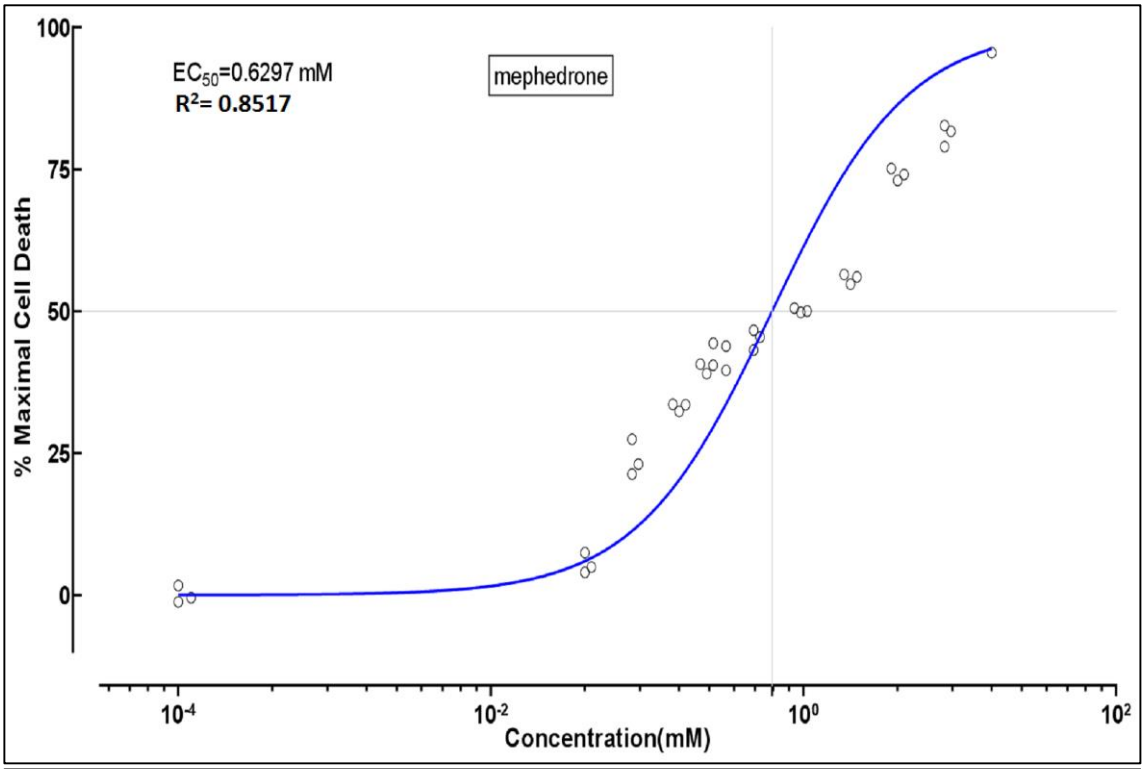
The potency of the drug to cause the effect is evaluated by EC_{50} value, which is defined as: 'the concentration of the agonist required to provoke a response halfway between the baseline and maximal response' (Motulsky and Christopoulos, 2004). In the current part of the work, the agonist is one of the selected NPS's and the provoked response is percentage cell death.

For cytotoxicity study using HepaRG™ cells, the data were collected from the three independent experiments using 11 different concentrations of each drug in the range of ($4.0x10^{-2}$-$1.6x10^{1}$ mM). The collected data about percentage of cell death were normalized on a scale of zero – µ%, where the blanks were considered as having zero percentage cell death. Adapted from normalization equation, the following equation was used to normalize the data on the desired scale of zero-100%:

$$\mathbf{Yn = Y/Ymax}$$

Where Yn: Normalized value, Y: original value and Ymax: maximum value in the series.

The collected data were plotted as percentage of cell death normalized to maximum response versus concentration, and the resultant normalized data were analysed using curve fitting with non-linear regression best-fit approach using 'GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, **www.graphpad.com [free trial]** '. The graph for all drugs showed increase in the response with increasing concentration (Figure 73)



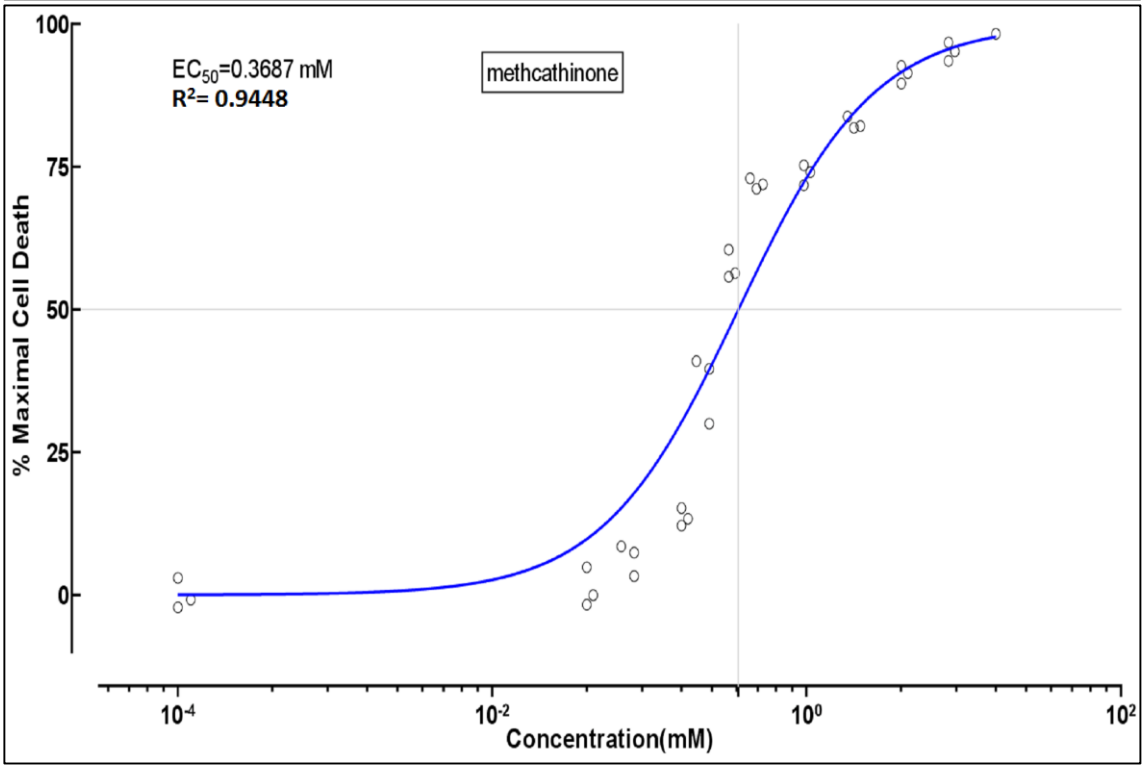


Figure 73 …... continued

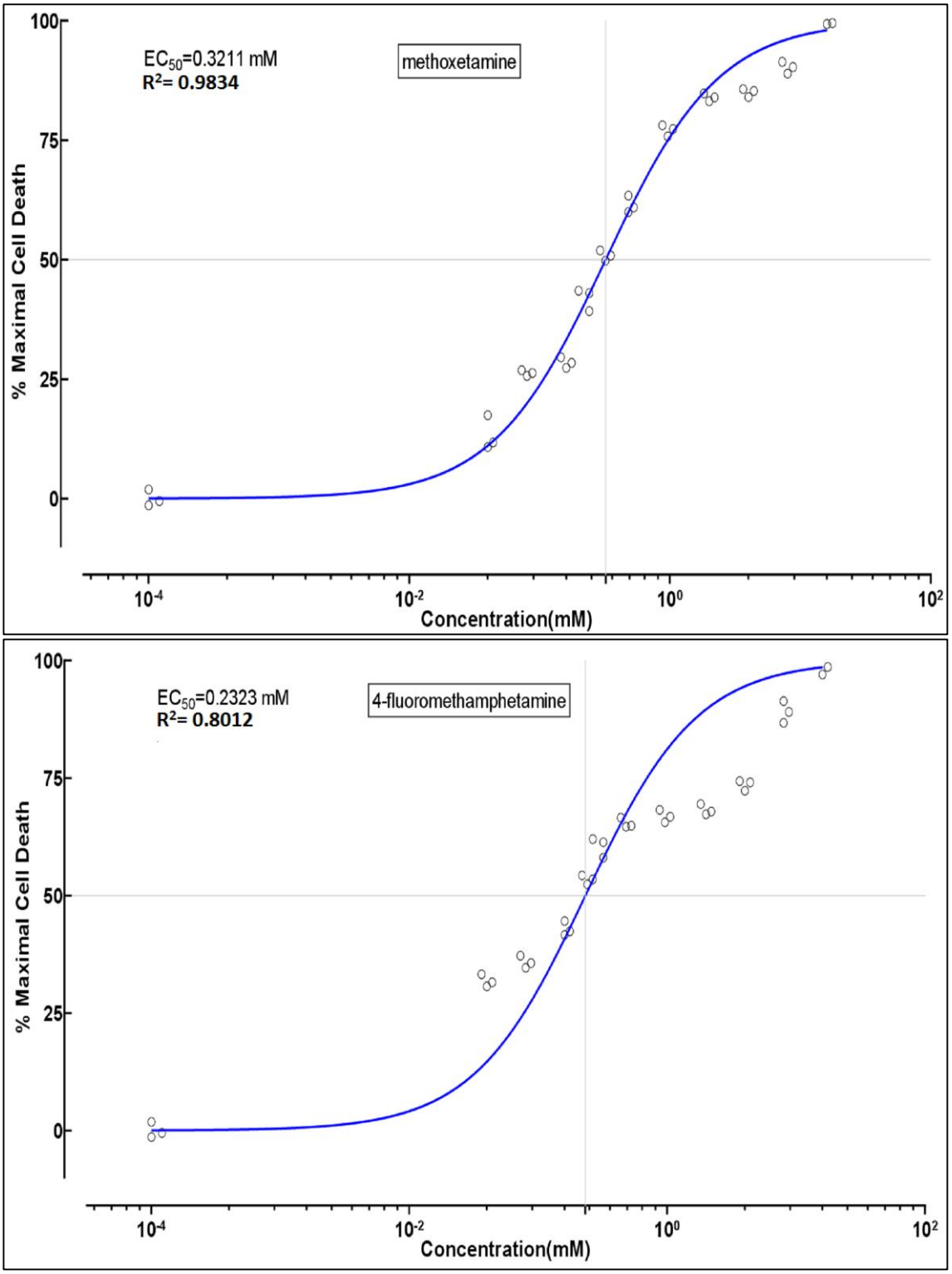


Figure 73: Normalized cell death percentage of HepaRG cells induced by selected NPS 48 h after incubation under specified conditions

All tested selected NPSs showed reproducible response that is dependent on concentration, where increasing the concentration increases the percentage of cell death. Within the concentration range used, 4MMC, 4FMA and MXE showed nearly similar average maximal percentage cell death of

about 84%, 78% and 73% respectively, while MCAT showed average maximal percentage cell death of about 45%.

In this part of the work, under the specified condition and within the specified range, 4FMA showed the most potent effect with EC_{50} value of 0.2323 mM (39 µg/ml). This is compared to MXE with EC_{50} value of 0.3211 mM (79 µg/ml), MCAT with EC_{50} value of 0.3687 mM (60 µg/ml) and 4MMC, which obtained the least cytotoxic profile, with $EC_{50\ value}$ of 0.6297 mM (111 µg/ml).

Compared to published data about cell death cytotoxic effect and other cytotoxic effects, the EC_{50} values of 4MMC, MCAT and 4FMA in the current work ranged between 0.2323 and 0.6297 mM. These values are lower than reported EC_{50} for amphetamines, cathinones, and related drugs in *in vitro* studies, where EC_{50} values ranged between 0.74 and 5.26 mM (Da Silva et al., 2013b). While no data is available for MXE or related drugs for the *in vitro* EC_{50} values for comparison The calculated molarity values were converted into the equivalent SI to compare with available reported toxic values through *in vivo*, post-mortem or clinical samples. Equivalent concentrations were 111, 60, 79 and 39 µg/ml for 4MMC, MCAT, 4FMA and MXE respectively (Table 19).

Those obtained EC_{50} values in this part of the work are many fold higher than the reported toxic peak concentration in either clinical intoxicated patients' samples or post-mortem samples. The reported concentration of mephedrone, as an example, in post-mortem biological samples in four fatalities in Scotland, ranged between 0.50-22 µg/mL (Torrance and Cooper, 2010). Compared to the obtained EC_{50} value of 0.6297 mM for mephedrone, which is equivalent to 111 µg/mL, is about 5-22 times more than the reported values in biological samples. In fact, it has been reported that tissue levels of amphetamine related drugs can be up to 18-30 times higher than blood concentrations. This discrepancy between *in vitro* and *in vivo* data, in case of amphetamines and related drugs, is partially due to their low protein binding affinity, which make their diffusion into tissues from plasma more favourable. In addition, post-mortem samples are most of the times from victims who have received emergency care in their pre-mortem intoxicated interval (Da Silva et al., 2013b).

In this part of the work, none of the selected NPS yielded 100% cell death. However, 4MMC, MCAT and MXE caused at least the death of 75% of the cells, while 4FMA at maximum used dose caused less than 50% of cell death. While one major effect– i.e. cell death – is studied here – is studied here, other cytotoxic effects with wider range needs further future studies to elaborate more about the toxic effects NPS in general, and the selected ones specifically (Table 19).

Table 19: MTT of selected NPS summary.

Drug	**Max cell death[1]**	**EC_{50} (mM)[2]**	**Equivalent [] (µg/ml)**	**Reported Toxic [] (µg/ml)[3]**
4MMC	84%	0.6297	111	0.5-22
MCAT	45%	0.3687	60	NA
MXE	78%	0.3211	79	NA
4FMA	73%	0.2323	39	NA

1: Max death cell induced under the specified condition and within the specified range ($4x10^{-2}$ – $1.6x10^{2}$ mM)
1: EC_{50} the concentration required to cause cell death a halfway between the baseline and maximal response
3: reported toxic values through *in vivo*, postmortem or clinical samples

Chapter 5 Summary and conclusion

The development of new analytical methods to detect NPS represents a significant challenge for forensic analysts, mainly due to the lack of reference standards and to the rapidly appearance of these diverse NPS. Moreover, their variability, easy availability and relatively easy synthesis, are all extra challenges to the authorities and scientific community. Most of these drugs are marketed as 'legal highs' or research chemicals to bypass the legal consequences. Most of the time, these chemicals cause many fatalities and morbidities before being legally framed as 'drugs of abuse'. In fact, the scientific community is the one responsible for developing novel approaches for the detection of these NPS at the earliest possible time and submitting solid data for the legal authorities at the point of appearance of these NPS. This responsibility is not limited to the analytical methods, but also anticipated clinical and toxicological profile studies that could provide evidence about the danger and possible risks of these chemicals. NPS are changing continuously, and the forensic chemists will be in the forefront of developing new analytical methods for detecting these drugs in forensic cases. Drugs of abuse from around the world are available nowadays through the internet.

Synthetic cathinones is a subgroup of NPS which is commonly abused for its euphoric effects, where Mephedrone, Methoxetamine, Methcathinone are examples. Upon performing an extensive literature survey, it was concluded that the currently available methods of analysis of these drugs and their metabolites in biological fluids did not fulfil the critical need of such methods in the field of forensic chemistry. 4-fluoromethamphetamine is an amphetamine-like drug of abuse with only one published method of analysis, and no metabolic studies, until now and to the best of our knowledge. NPS are commonly traded as mixtures, so our decision to analyse the drugs of interest concomitantly may be helpful for analysts who are interested in the analysis of these drugs and any other related drug.

In the first part of the thesis, HPLC was utilized for qualitative and quantitative analysis of mephedrone, alone and concomitantly with methcathinone. A method was developed and fully validated for analysis by HPLC-DAD after LLE from spiked blood and serum samples. The LLE protocol was optimized by experimentation of different extraction solvents and pH modifications to avoid emulsion formation which is a problem often appeared in this type of extraction. LLE

efficiency was improved reflected in the satisfactory validation parameters. The method showed simplicity to apply, robustness, repeatability, rapidity and validity over the specified range. The analytical method was shown to be linear over the specified range of 0.1-10 µg/ml, repeatable with RSD values of 3.37-3.98% and 4.05-6.45% for intraday and intraday repeatability respectively, with accuracy represented by extraction fraction of 83-109%, specific for the analytes and with LOD and LOQ of 0.011-0.014 and 0.036-0.043 µg/ml respectively. These values are comparable to those published data utilizing HPLC-DAD or other analytical techniques. For example, LOQ in one study for mephedrone in biological fluids was determined to be 0.1 µg/mL (Torrance and Cooper, 2010), and in another study LOD and LOQ were determined to be 0.010 and 0.025 µg/mL respectively (Dickson et al., 2010). The values are comparable to published similar HPLC-DAD data for analysis of mephedrone after LLE from blood, where LOD and LOQ were determined to be 0.039 and 0.078 µg/mL respectively (Maskell et al., 2011). The working range is also comparable to other published data and cover the range of observed clinically toxic concentration. In conclusion, the use of solvent mixture with the addition of alcohol seems to work more efficiently for the extraction of drugs from biological samples. In addition, due to the basic nature of most of drugs of abuse, we buffered the samples with a buffer solution of pH 10, as this will present the drugs in their non-ionisable form. Similarly, important, this will minimize emulsion formation, where extreme pH seems practically to be one of the predisposing factors. The developed valid HPLC-DAD analytical method for the quantitative analysis of mephedrone alone and with methcathinone presented in this chapter can be considered as a reference argument for future analysis of these designer drugs and any related drugs utilizing such instrumentation.

In the second part of thesis, *in vitro* studies on the metabolism of selected NPS using pig liver microsomes and a qualitative LC/MS analysis were successfully performed. Liver microsomes were prepared by a conventional ultracentrifugation method and used for performing metabolism of the selected drugs. The *in vitro* study was applied to mephedrone and methcathinone as a continuation of the previous work. MXE was added after it appears just as this *in vitro* work was started, using liver microsomes. Samples were analysed utilizing LC-MS under the specified conditions and parent drugs and detected metabolites separated with good resolution in less than10 minutes total run time.

The products of the metabolic study were prepared by filtration and injected onto LC-MS. After initial runs under M/S conditions, chromatograms were extracted for the molecular ions of the parent drug and expected metabolites. Two of the detected peaks from the MS study were suggestive as being metabolites for the drug mephedrone, while another two were suggestive as being metabolites for the drug MXE.

Detected mass ions were then analysed under MS/MS conditions using SIM and SRM mode. The produced chromatograms after MS/MS analysis showed peaks in concordance with detected peaks of the MS study. Mass spectra of the detected peaks were confirmative for the identity of the suggested metabolites of mephedrone and for one of the suggested metabolites of MXE, while other peaks were not inclusive for the identity of other suggestive metabolite of MXE.

The detected peaks for mephedrone metabolism were those of the parent drug (MH+=178), one with MH+=194 and another peak with MH+=166. The detected peak with MH+194 was suggested to be either hydroxytolyl-mephedrone as a product of hydroxylation of the tolyl moiety or 4-carboxy-nor-mephedrone as a product of demethylation and two steps of hydroxylation. Comparative fragmentation pattern of both possible metabolites with the detected fragments after MS/MS analysis using SRM mode were confirmative for the identity of the detected metabolite to be hydroxytolyl-mephedrone. The other detected with MH+=166 was suggestive to be dihydro-nor-mephedrone as a product of two steps of N-demethylation and reduction of the ketone moiety. Compared to the expected fragmentation pattern, with the detected fragment after MS/MS was inclusive for the identity of the other metabolite to be dihydro-nor-mephedrone. None of the other suggested metabolites were detected, most probably due to the presence of these metabolites as intermediates or in concentration below the limit of detection. These findings are consistent with previously and recently published data about the detected metabolites of mephedrone as presented by Meyer et al. and Pedersen et al., though more numbered metabolites were reported. Meyer et al. reported both metabolites presented here, while Pedersen et al. reported only hydroxytolyl mephedrone but not the dihydro-nor-mephedrone. To the best of our knowledge, the detected metabolite dihydro-nor-mephedrone was only reported by Meyer et al. as through *in vivo* study, while its reported here in an

in vitro study (Meyer et al., 2010; Pedersen et al., 2013). Though variation of the *in vivo* and *in vitro* system are taken into consideration, these findings are supportive for the power of *in vitro* metabolic studies as a predictive tool for the metabolic profile of NPS.

For MXE, the detected peaks were those of the parent drug (MH+=248), one with MH+=234 and another peak with MH+=250. The detected peak with MH+=234 was suggestive to be O-desmethyl MXE as product of O-demethylation Compared to the expected fragmentation pattern, with the detected fragment after MS/MS, data were confirmative for the identity of the metabolite to be O-desmethyl MXE. The other detected peak with (MH+=250) was suggestive to be either dihydro-MXE or O-desmethyl -hydroxy--MXE. Comparative fragmentation patterns of both possible metabolites with the detected fragments after MS/MS analysis using SRM mode were not conclusive for the identity of the molecule.

For methcathinone, we were not able to detect any of the expected metabolites. Most probably none were produced. In fact, potentially and theoretically methcathinone would be metabolised in a similar way to mephedrone. Limited data are available about the metabolism of methcathinone for comparison.

Tandem MS analysis was helpful to support the proposed fragmentation pattern of the parent drugs and detected metabolites. Mass spectrometry and LC/MS in particular is a powerful tool for identification and structure elucidation, compared to the limited application of LC/DAD instrumentation for elucidation purposes. Analysis showed successful *in vitro* metabolism and microsomes preparation, demonstrated by the production of hydroxytolyl-mephedrone and nor-dihydro mephedrone. For MXE, two first-time reported metabolites were presented, produced by the O-demethylation and the reduction of the ketone moiety to the corresponding alcohol, respectively.

The third part of the work moved to another level of *in vitro* studies utilizing hepatocytes. To complete the spectrum of the most frequently used analytical instrumentation in forensic laboratories, GC-MS was utilized for the analysis of selected NPS and potentially produced metabolites. As a continuation of the previous work, the *in vitro* studies were applied to mephedrone, methcathinone

and methoxetamine. 4-fluoromethamphetmine, which appeared as a NPS when this work commenced was added to the analysis. Up to the present time, no similar data are available about the metabolic profile of 4-fluoromethamphetamine. In addition, up to the present time, no similar data about cytotoxic effects of any of the selected NPS are available.

Studying the selected NPS utilizing GC-MS, mephedrone and methcathinone showed nearly similar fragmentation patterns, comparable to each other and to the confirmed fragmentation pattern of cathinones. The fragmentation pattern described here is relatively similar to the published data, specifically about mephedrone. However, we did not find a similar description for the fragments produced by the loss of N-methyl group producing (M+ -15) fragments.

For mephedrone and methcathinone, an additional peak appeared in both cases with base peak of 56. Previous published data described the appearance of *m/z*=56 as a consequence of loss of H_2 of the iminium ion. However, the suggestion is that the identification of a molecule with base peak of *m/z*=56 is truly due to net loss of the loss of H_2 and formation of ketoimine molecule at some stage through analysis, but not as part of the fragmentation pathway. The fragmentation pattern of methoxetamine is similar to previously published data, while no similar data for comparison was available for 4-fluoromethamphetamine.

Utilizing HepaRG and hepatocytes for metabolic studies, we were able to identify the parent molecules utilizing GC-MS. It was possible to detect new peaks when studying the chromatograms of mephedrone and methcathinone, where in both cases the mass spectra of theses peaks were However, we were unable to identify any of the potential metabolites or identify any of the analysed peaks to be of the metabolites. Compared to previous work using microsomes as an *in vitro* metabolic system, we were able to detect metabolites of some of the selected drugs, indicating success of this *in vitro* model. However, we could not detect any metabolites using the purchased primary human hepatocytes and HepaRG cell lines. That is most probably due to the previously discussed drawbacks of these *in vitro* metabolizing models.

Primary human hepatocytes, being currently isolated from surgically dissected liver with tumour or other liver diseases leads to deficiency of some liver specific functions, especially P450 enzymatic activity and also they may lose some of their metabolizing functions when they are in the suspension form as they lose their naturally occurring polarity. HepaRG cell lines which are originally taken from a liver tumour of a human donor with chronic hepatitis C also has limitations, mainly being of a particular genotype affecting the type of produced drug-metabolizing enzymes. Some genotypes such as CYP1A2, CYP2A6 and even CYP2D6 are poorly expressed in HepaRG cells which in turn leads to reduction in their metabolic capacity of the selected drugs.

HepaRG cell line was utilized for the assessment of cytotoxic effects of the selected NPS, demonstrated by cell death. Collected data showed that, within the specified range, 4FMA showed the most potent effect with EC_{50} value of 0.2323 mM (39 µg/ml) while mephedrone showed the least toxic effects with EC_{50} value of 0.6297 mM (111 µg/ml). This is compared to methoxetamine with EC_{50} value of 0.3211 mM (79 µg/ml) and methcathinone with EC_{50} value of 0.3687 mM (60 µg/ml). No similar data was available for comparison of methoxetamine toxic effects, while for other drugs, results were comparably lower than reported EC_{50} for amphetamines, cathinones, and related drugs in *in vitro* studies.

Future work

Drugs abuse is a serious and historical problem that needs continuous close observation and multidisciplinary work to dry its resources. The sharp skills and knowledge acquired through this research together with my medical background and close work with drug abuse prevention authorities in my home country are believed to strengthen my intention and future plans to integrate all efforts and focus them toward the benefit of society.

As an of the thesis research work, further research work is needed to keep pace with the rapidly appearing 'NPS'. Success of the *in vitro system* as a predictive tool for the toxicological profile of NPS, either through detecting the metabolites or their cytotoxic effects, mandate more consideration and optimization for this kind of non-invasive, relatively cheap, informative and ethically acceptable approach. Though this kind of approach is well established in the pharmaceutical industry compared with their use in forensic sciences, they are not less useful for the prediction of clinical and toxicological profiles of drugs of abuse.

Through this thesis, the phenomenon of keto-enol tauterism was raised as an issue when analysing the selected beta-ketoamine selected NPS using GC-MS. Keto-enol tauterism is well discussed in literature, but limited information is available about it regarding drugs of abuse in general. Thermal instability of these molecules was an issue raised as well, represented by production of different, yet comparable, fragmentation patterns. The importance of this phenomenon is the different fragmentation pattern that will arise which may –if not considered – affect the interpretation of data and quality of results. Further studies are needed to estimate and quantify the effect of these phenomena for the selected NPS and related NPS.

In vitro cytotoxicity studies using HepaRG cell lines were informative about the cytotoxic effects that selected NPS may have on cells resembled by cell death. This type of studies is helpful as preliminary evaluation step. Future studies may include study of the cytotoxic effects selectively on different type of cells (i.e. brain, liver, etc. …) as a predictive tool for local and systematic effects of NPS.

Wide selection of NPS is intended, with continually updating the list of included drugs to keep pace with newly appearing one. This climate of the emergence of new drugs of abuse mandates robust, easy to prepare and relatively cheap optimised analytical approaches.

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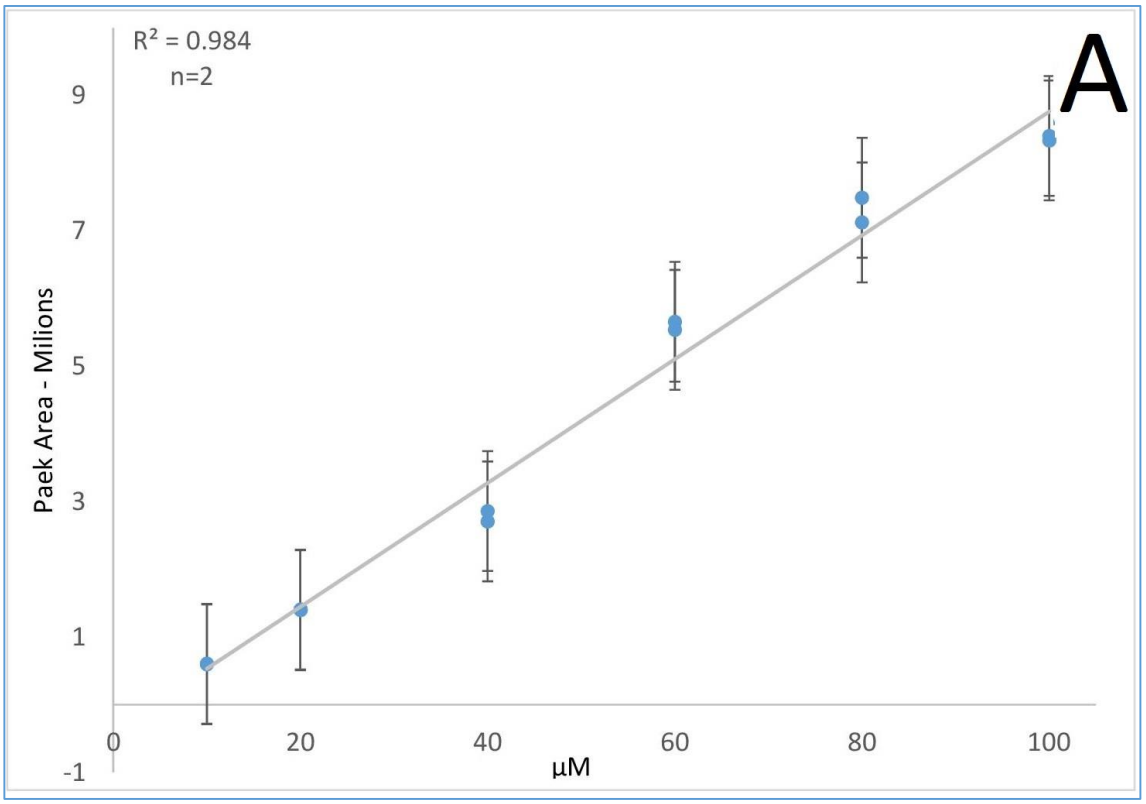
Appendix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Paek Area - Milions
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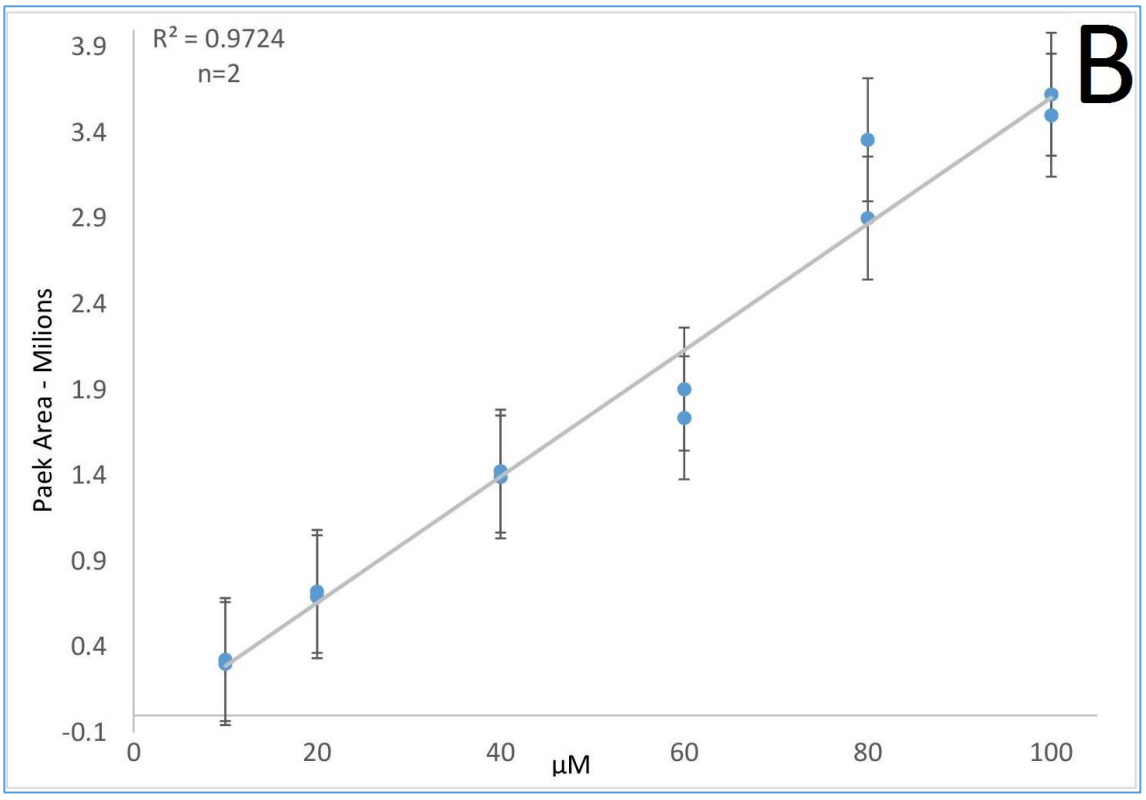
7
5
3
1
-1
0
20
40
60
80
100
µM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Paek Area - Milions
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3.4
2.9
2.4
1.9
1.4
0.9
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0
20
40
60
80
100
µM

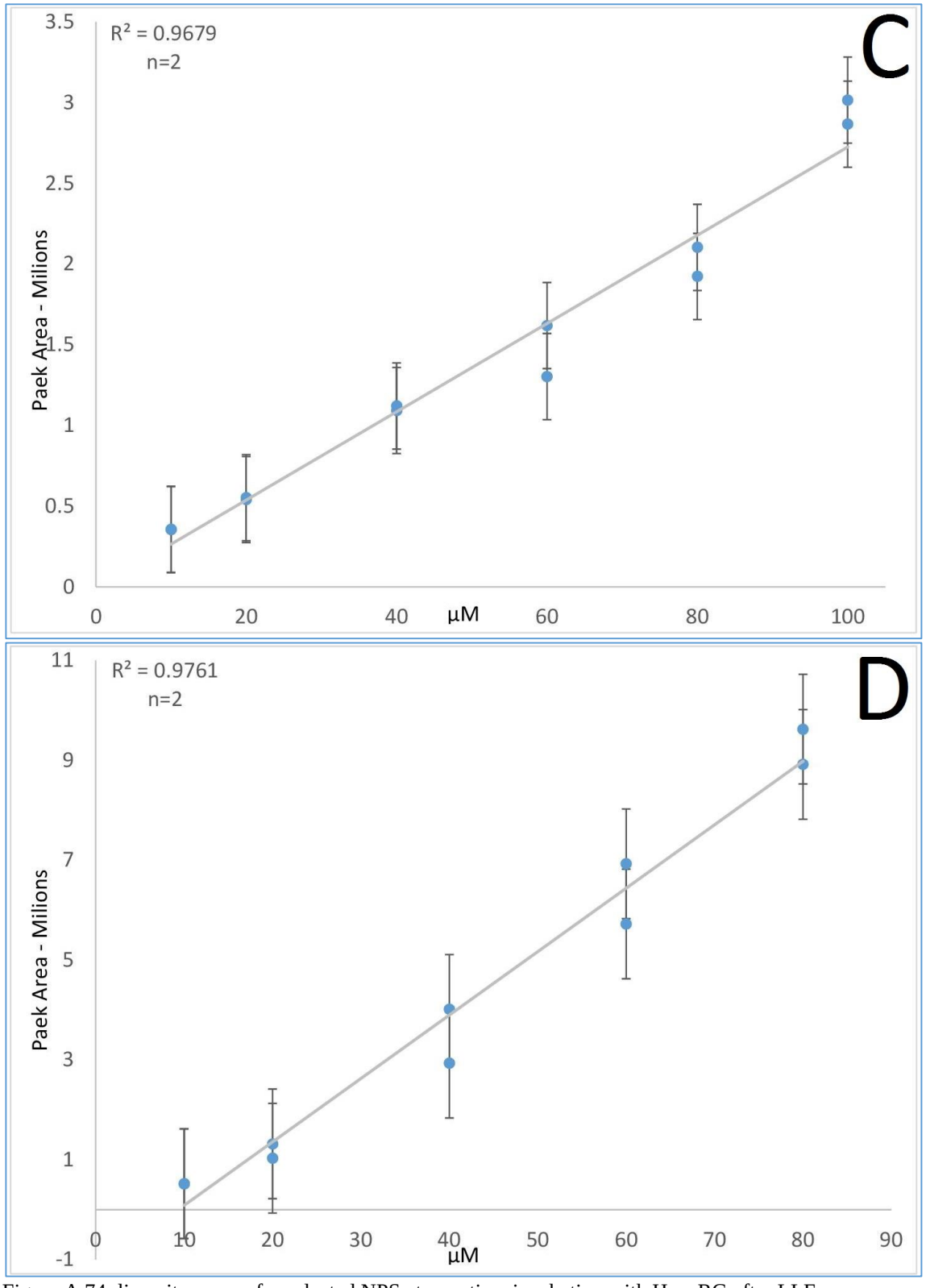

Figure A.74: linearity curves for selected NPS at zero time incubation with HepaRG after LLE.

Poster presentation in the 22nd Congress of the International Academy of Legal Medicine (IALM 2012) . Istanbul – Turkey . July 5-8,2012.

[PP-442]

Simultaneous analysis of mephedrone and methcathinone after liquid-liquid extraction and analysis using HPLC-DAD

Majed Yasin Alshamaileh, Jose Gonzalez Rodriguez, Mark Baron, Ruth Croxton
University of Lincoln

The cultural problem of drugs abuse is of critical importance for the public and the authorities. Beta-ketoamphetamines, with methcathinone and mephedrone (4-methylmethcathinone) as best known members of this family, are ones of the most commonly experienced "legal highs". Mephedrone, one of the newly controlled 'legal highs' is a synthetic cathinone derivative, where its history dates back to 1929. Not much data is available about the clinical or toxic effects of the mephedrone. Mephedrone started to appear online and become available in 2007, and it was late April 2010 till mephedrone was added to the list of the illegal drugs of abuse in the UK. Methcathinone is a methyl derivative of cathinone which has similar clinical effects to Amphetamine, to its methylated form (i.e.: mephedrone) and to cathinone. A method for the liquid-liquid extraction (LLE) and simultaneous analysis of the drug mephedrone with the drug methcathinone was developed and validated. LLE of these drugs from biological samples (whole blood or serum) and reverse phase HPLC-DAD were optimized for qualitative and quantitative analysis. Linearity of the method was obtained with R2>0.99 for both drugs over the specified range (0.1-10 µg/mL). The accuracy was assessed by calculating percentage recovery at different concentrations for both drugs. The method was found to be accurate with recovery values for both drugs ranging between 83-109%. For repeatability and intermediate precision tests, RSD values were <= 8.4%. The method was found to be specific for both drugs with a limit of quantitation (LOQ) ranging between 0.036 and 0.043 µg/mL and limit of detection (LOD) ranging between 0.011 and 0.013 µg/mL for mephedrone and methcathinone, respectively.

Keywords: mephedrone,methcathinone,HPLC-DAD, Liquid-Liquid extraction

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[OP-120]

In-vitro metaboilsm and analysis of Legal Highs using LC-MS

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University of Lincoln

The cultural problem of drugs abuse is of critical importance for the public and the authorities. "Designer drugs" are those compounds produced by performing minor alterations to one or more functional groups of a known chemical with specific pharmacological activity, to avoid the legal regulations and to produce more effective substances. In the latest few decades a new class of "designer drugs" known as "legal highs" has emerged on the abuse drugs market, mostly through internet websites. As "legal highs" appear, usually, for a short while before they have been controlled, no much data could be known about their metabolic pattern. At the time they appear on the market the metabolism of these drugs is generally unknown, and therefore, it must be studied in order to obtain data necessary for analytical method development as well as toxicological risk assessment. In-vitro metabolic studies are well established in the pharmaceutical industry; however, no much work has been performed in the field of forensic toxicology. In-vitro metabolism studies of new designer drugs can be done for qualitative and quantitative assessment of new designer drug metabolites. In our research work, liver and lung microsomes and S9 fractions were prepared in our laboratories, and used to assess the metabolic pattern of 'legal highs'. The tested 'Legal highs' were bought form internet-based companies; and their identities were confirmed by mass spectral study. Evaluation of the metabolism of mephedrone, MDAI, methcathinone and methoxetamine were performed, and up to the point, the prepared microsomes and S9 fractions system proved to be functioning to metabolise some of the mentioned 'legal highs', where they and their metabolites were analysed using LC-MS/MS.

Keywords: Legal highs, microsomes, S9

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